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(71) Applicant (*for all designated States except US*): CHILDREN'S HOSPITAL & RESEARCH CENTER AT OAKLAND [US/US]; 747 52nd Street, Oakland, CA 94609 (US).

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): MORRIS, Claudia R. [US/US]; 1595 Huston Road, Lafayette, CA 94549 (US).

(74) Agent: FRANCIS, Carol L.; BOZICEVIC, FIELD & FRANCIS LLP, 200 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).

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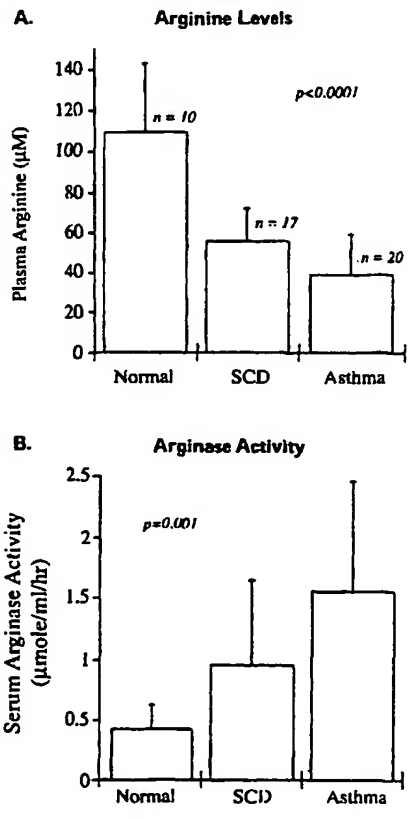
(54) Title: TREATMENT OF CONDITIONS ASSOCIATED WITH DECREASED NITRIC OXIDE BIOAVAILABILITY, INCLUDING ELEVATED ARGINASE CONDITIONS



(57) Abstract: The invention features methods and compositions for treatment of conditions associated with decreased nitric oxide bioavailability, such as a condition associated with elevated arginase activity, using an arginine-based therapy, including combination therapy with an arginase inhibitor and/or magnesium.

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TREATMENT OF CONDITIONS ASSOCIATED WITH DECREASED NITRIC OXIDE BIOAVAILABILITY, INCLUDING ELEVATED ARGINASE CONDITIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the priority benefit of U.S. provisional application serial no. 60/447,373, filed February 14, 2003, which application is incorporated by reference herein in its entirety.

GOVERNMENT RIGHTS

[0002] This invention was made with government support under federal grant nos. RR01271-19 and HL-04386-01 awarded by the National Institutes of Health. The United States Government may have certain rights in this invention.

FIELD OF THE INVENTION

[0003] The present invention is in the field of therapy for conditions associated with elevated arginase as described herein, including asthma, sickle cell disease, and pulmonary hypertension.

BACKGROUND OF THE INVENTION

[0004] L-Arginine (Arg) is a conditionally essential amino acid, naturally found in dietary protein. It is converted to nitric oxide (NO) (1, 2) a potent vasodilator (1-3) and bronchodilator (4, 5), by a family of enzymes known as nitric oxide synthase (NOS). NO is an essential molecule that plays a role in a broad range of functions from vascular regulation, neurotransmission (2), host defense, and cytotoxicity (6) to physiologic control of airways (5). Under conditions of low L-arginine concentration, nitric oxide synthase is uncoupled and reduces oxygen (O_2) to superoxide (O_2^-) instead of generating nitric oxide (7,8). Nitric oxide reacts rapidly with superoxide to form reactive nitric oxide species (*RNOS*) that could lead to worsening inflammation, oxidative stress and cellular damage (9).

[0005] Complex interactions among cellular components of the immune system, endocrine factors, growth factors and cytokines contribute to pathophysiology of asthma. The contribution of some cytokines (IL-1α, IL-1β, IL-3, IL-4, IL-6, IL-11, TNF-α, γγ- interferon, IL-6, M-CSF) have been well studied in asthma. Secretory phospholipase A2 (sPLA2), which is involved in the pathway of leukotriene synthesis is elevated in broncheoalveolar lavage of

antigen-challenged asthmatics (10). The genetic predisposition of asthma is now well recognized (11).

[0006] Recently, expression of inducible NO synthase, the enzyme that catalyzes the production of NO from L-Arg, has been found in the epithelium of asthmatic patients but not in healthy non-asthmatic patients. (12, 13). Asthmatics have exhaled air NO levels that are 3.5 times higher than non-asthmatics, which are correlated with decrease in FEV_1 and are affected by therapy (14). Blocking of NO production by L-Arg analogues results in an increase in allergen-induced bronchoconstriction (15). A deficiency of NO is involved in airway hyperreactivity (16). Although asthma is clearly a multifactorial disease, there is some evidence that NO may play an important role in disease pathogenesis (17). For reviews, see, e.g., Dweik Cleve Clin J Med. 2001 Jun;68(6):486, 488, 490, 493; Gianetti et al. Eur J Clin Invest. 2002 Aug;32(8):628-35.

[0007] Arginase is an enzyme that hydrolyzes Arg to produce ornithine and urea, (35) however, in the presence of nitric oxide synthase (NOS), arginine is converted to nitric oxide (NO) and citrulline (2). The expression of arginase can be induced by a variety of cytokines involved in the inflammatory process (26), particularly the Th2 cytokines. (37). Increased serum arginase activities have been reported in patients with SCD at steady-state (38), as well as in an asthma animal model (39). Arginase activity is elevated in SCD patients with pulmonary disease (34, 40). Plasma arginase activity appears to be related to hemolysis, associated with several markers of hemolytic severity, including LDH ($r=0.44$, $p<0.001$), AST ($r=0.39$, $p<0.002$), reticulocyte count ($r=0.25$, $p<0.001$), and Hct ($r= -0.25$, $p<0.001$) (Morris et al, Erythrocyte arginase release during hemolysis contributes to endothelial dysfunction and pulmonary hypertension, 27th Annual Meeting of the National Sickle Cell Disease Program, Los Angeles, CA; April 2004).

[0008] Arginase controls the metabolism of arginine into ornithine, which in turn gives rise to proline and polyamines (37, 41-43). These downstream products of arginase activity may play a significant role in the pathogenesis of asthma, pulmonary hypertension and other inflammatory conditions, since proline is involved in collagen formation (44, 45) and lung fibrosis (46), processes that occur in airway wall thickening and airway remodeling (47-50).

[0009] Elevated levels of polyamines have been reported in the serum of asthmatic patients (51). Polyamines have contractile activity on smooth muscle (52, 53), and are present in multiple cell types in the lung, including airway epithelium, smooth muscle cells and macrophages (54). Since proline and hydroxyproline (47) are amino acids involved in collagen deposition, and polyamines affect multiple processes, including cell survival, cell proliferation and mucus production (52, 53), they may play a role in lung pathology.

[0010] Arginine, a safe dietary supplement, has already demonstrated potential for therapeutic utility in several disease processes.(18-23). In animal studies, inhalation of low doses of L-Arg has completely blocked hyperresponsiveness of reactive airways (13, 24), and inhaled L-Arg also improves pulmonary functions of cystic fibrosis patients (CF) (25,26). When tested in a mouse model of allergic asthma, oral administration of L-Arg was reported to aggravate allergen-induced eosinophilic airway inflammation (Takano et al. J Pharmacol Exp Ther 1998 Aug;286(2):767-71).

[0011] Use of L-Arg is suggested for treatment of cystic fibrosis (Busch-Petersen et al. Z Erkr Atmungsorgane 143:140-7 (1975)); treatment of exercise induced pulmonary hemorrhage in horses (US Pat. No. 6,027,713); and treatment of pulmonary hypertension (U.S. Pat. Nos. 5,217,997; 6,127,421; Nagaya et al. Am J Respir Crit Care Med 163:887-81 (2001); Cheng et al. Hua Xi Yi Ke Da Xue Xue Bao 27:68-70 (1996)).

[0012] Use of NO to treat asthma is discussed in Nakagawa et al. J Pediatr. 2000 Jul;137(1):119-22;and Rossaint et al. Eur Heart J 1993 Nov;14 Suppl I:133-40). The arginase inhibitor N-hydroxy-L-arginine (NOHA) has been tested in a model of asthma (see, e.g., Meurs et al., Br J Pharmacol Jun 2002, 136(3):391-8, describing administration of an arginase inhibitor in a guinea pig model of allergic asthma; and Meurs et al. Br J Pharmacol 130:1793-8 (2000, describing arginase inhibitors in a perfused guinea pig trachea model)). Use of NO to treat pneumonia has been discussed (see, e.g., Kimura et al. Pediatr Int 2002 Aug;44(4):451-2; Ho et al. J R Soc Med 2002 Jan;95(1):35-7; Bugge et al. Eur J Anaesthesiol 2000 Apr;17(4):269-72; Hoehn et al. Respiration 1998;65(6):477-80; Blomqvist et al. Acta Anaesthesiol Scand 1993 Jan;37(1):110-4; Jean et al. Crit Care Med 2002 Feb;30(2):442-7 and Kannan et al. Indian J Pediatr 1998 May-Jun;65(3):333-45).

[0013] Although early investigators warned of the deleterious impact of nitric oxide in sickle cell disease (SCD) (27), more recent studies support its protective function (28). Similar to asthmatic patients (29), SCD patients also have elevated NO_x levels at baseline (30). Serum L-Arg and NO_x levels fall, however, during the vaso-occlusive complications of SCD, (31) with lowest levels found during acute chest syndrome (pneumonia). Most SCD patients with pulmonary disease have a component of reactive airways that respond to bronchodilators, even though they often do not demonstrate the classical wheezing on physical exam that is usually associated with asthma. Asthma in SCD is often unrecognized and undertreated, and occurs in 30-60% of patients (32). Clinical trials of arginine therapy are now underway for SCD (33, 34).

[0014] Magnesium, which can be a dietary supplement, has been described as an adjuvant in combination therapy of asthma with salbumatol (Hughes et al, Lancet 2003;361:2114-7) or as

an asthma intravenous monotherapy (Gurkan et al, Eur J Emerg Med 1999;6:201-5). Magnesium has also been suggested in infusion therapy of neonatal pulmonary hypertension (Patole S & Finer N, Magnes Res 1995;8:373-88). The effects of oral magnesium in an animal model of pre-eclampsia has been reported (Pandhi et al, Indian J Exp Biol 2002;40:349-51) and other disease processes that involve endothelial dysfunction (Volpe et al, Scand Cardiovasc J 2003; 37:288-96). Magnesium-induced vasodilation has been reported in animal models of other conditions that involve endothelial-derived nitric oxide (Teragawa et al, Magnes Res 2002;15:241-6, describing the effects of magnesium in an in vitro canine coronary artery model of endothelial dysfunction). Combined therapy of magnesium and inhaled nitric oxide has shown some promise in an animal model of pulmonary hypertension (Haas et al, Pediatr Int 2002;44:670-4).

[0015] Despite the advances in the field with respect to therapies for conditions such as asthma and sickle cell disease, new therapies are of considerable interest and importance. Furthermore, therapies based upon a more insightful understanding of the underlying mechanisms of these diseases is needed so as to provide a more rationale approach to therapy.

[0016] There is a need in the field for improved or alternative therapies for treatment of conditions such as asthma. The present invention addresses these needs.

Literature

[0017] U.S. Pat. Nos. 5,217,997; 6,387,890; 4,507,314; 6,359,007; 6,646,006; 6,165,975.

[0018] American Society of Hematology Meeting, San Diego Dec 2003; Morris et al, Blood 2003;102:763a (abstr2818); Inselman et al. "Alterations in plasma amino acid levels in children with asthma: a preliminary investigation." Pediatr Pulmonol. 1986 May-Jun;2(3):163-9; Jorens et al. "L-arginine-dependent nitric oxide synthase: a new metabolic pathway in the lung and airways." Eur Respir J. 1993 Feb;6(2):258-66; Vercelli "Arginase: marker, effector, or candidate gene for asthma" J Clin Invest. 2003 Jun;111(12):1815-7 and Zimmermann et al. "Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis." J Clin Invest. 2003 Jun;111(12):1863-74 relate to microarray analysis of the expression profiles of lung tissue in two murine models of asthma revealed high levels of arginase I and arginase II activity, in association with IL-4 and IL-13 overexpression. Haas et al, "Nitric oxide further attenuates pulmonary hypertension in magnesium-treated piglets" Pediatr Int 2002;44:670-4.

[0019] Meurs et al. "Arginase and asthma: novel insights into nitric oxide homeostasis and airway hyperresponsiveness." Trends Pharmacol Sci. 2003 Sep;24(9):450-5 provides a review

in which the authors proposed that a relative deficiency of NO caused by increased arginase activity and altered L-arginine homeostasis is a major factor in the pathology of asthma.

[0020] Sapienza et al. "Effect of inhaled L-arginine on exhaled nitric oxide in normal and asthmatic subjects." Thorax. 1998 Mar;53(3):172-5 reports that inhaled L-Arg increased exhaled NO in a dose-dependent fashion, with the cumulative effect of L-arginine on NO in asthmatic subjects being significantly higher than in non-asthmatics. This report concluded that L-Arg may have therapeutic potential in diseases in which there is defective production of NO, but in asthma it may amplify the inflammatory response in the airways.

[0021] De Gouw et al. "Effect of oral L-arginine on airway hyperresponsiveness to histamine in asthma." Thorax. 1999 Nov;54(11):1033-5 concludes that oral L-arginine does not influence airway hyperresponsiveness to histamine as reflected by PC(20), although the dose-response slope is slightly reduced in patients with asthma, thus indicating only marginal, clinically unimportant limitation of NO synthase substrate in asthma.

[0022] Chambers et al. "Effect of nebulised L- and D-arginine on exhaled nitric oxide in steroid naive asthma." Thorax. 2001 Aug;56(8):602-6. reported that administration of inhaled L-Arg to asthma patients induced bronchoconstriction, with Exhaled NO decreasing with acute bronchoconstriction, and returning to baseline with the resolution of bronchoconstriction. Exhaled NO increased following the administration of both L-arginine and D-arginine.

SUMMARY OF THE INVENTION

[0023] The invention features methods and compositions for treatment of conditions associated with decreased nitric oxide bioavailability, such as a condition associated with elevated arginase activity, using an arginine-based therapy, including combination therapy with an arginase inhibitor and/or magnesium.

[0024] The invention is advantageous in that, where the invention contemplates administration of arginine in combination with an arginase inhibitor, the invention can avoid the need to administer higher doses of arginine that may otherwise be needed to treat conditions associated with elevated arginase activity. In short, where elevated arginase increases utilization of arginine, higher doses of arginine would be required to overcome this phenomenon in an arginine monotherapy. Administration of an arginase inhibitor in conjunction with arginine can lower therapeutic dose requirements of arginine. A large dose of arginine, e.g., up to 10 pills, three times a day, that may otherwise be required without combination therapy with an arginase inhibitor is a very large hindrance to achieving therapeutic goals, largely due to poor patient compliance.

[0025] Administration of arginine to a patient having elevated arginase levels leads to increased production of ornithine. Plasma ornithine levels strongly correlated to proline levels in asthmatic patients ($r = 0.75$, $p<0.0001$, $n = 26$) . The administration of an arginase inhibitor together with arginine will have the added benefit of decreasing the downstream by-products of ornithine metabolism, e.g., proline and polyamines, both of which are associated with pulmonary and cardiovascular pathology through airway remodeling, lung fibrosis and vascular smooth muscle proliferation. This invention will provide substrate for nitric oxide production, while limiting production of metabolites of arginase activity that would otherwise likely contribute to disease pathology.

[0026] Ornithine also decreases arginine bioavailability through competitive inhibition since arginine and ornithine use the same transporter molecules. In short, elevated arginase activity decreases arginine bioavailability. Arginine administered with an arginase inhibitor maximizes arginine bioavailability even in the context of elevated arginase levels.

[0027] Still another advantage of the invention is that, compared to administration of arginase inhibitor alone, is that arginase inhibitors are quite expensive. Administration of arginine, which is relatively inexpensive, in conjunction with an arginase inhibitor allows for administration of relatively reduced amounts of expensive arginase inhibitors. In short, administration of arginine and arginase inhibitors will be more effective, and a less expensive therapy.

[0028] Another advantage is that the invention avoids the problem that arginine bioavailability remains limited by its low concentration, even in the presence of an arginase inhibitor. Low arginine concentration leads to the uncoupling of nitric oxide synthase (NOS) and superoxide production in lieu of nitric oxide. The Km for arginine transport on the cationic amino acid molecules is around 100 μM; thus reversing the arginine deficiency while maximizing arginine bioavailability and limiting alternate routes of metabolism as per the present invention provide for an improved means for achieving therapeutic goals.

[0029] These and other advantages will be apparent to the ordinarily skilled artisan upon reviewing the present specification.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] Figure 1 is a graph showing plasma Arginine concentration (Panel A) and arginase activity (Panel B) in normal non-asthmatic controls (*Normal*, n=10) vs. SCD patients with PHT (*SCD*, n=17), vs. patients with asthma (*Asthma*, n=20). Arginine levels are low and arginase

activity is elevated in patients with asthma and in SCD patients with pulmonary hypertension compared to normal controls ($p < 0.0001$).

[0031] Figure 2 is a graph showing the change in plasma arginine levels from initial presentation to the emergency department (*Admit*) vs. the day of hospital discharge (*D/C*) in asthmatic children (four patients) requiring hospitalization. Low arginine levels rise significantly as clinical condition improved ($p \leq 0.05$).

[0032] Figure 3 is a graph demonstrating changes in plasma arginine and ornithine concentration (Panel A; closed circles, arginine levels; open circles, ornithinine levels) , arginase activity and nitric oxide metabolites (Panel B; closed circles arginase activity; open circles, serum nitric oxide metabolites (NOx)) during hospitalization in a representation four-year old boy with status asthmaticus.

[0033] Figure 4 is a schematic illustrating competition of arginase with nitric oxide synthase for available L-arginine substrate. Downstream by-products of arginase activity are compounds that likely contribute to disease pathogenesis.

DEFINITIONS

[0034] "Arginine" or "Arg" or "L-Arg" as used herein refers to naturally occurring or synthetically produced L-arginine.

[0035] "Arginase" as used herein refers to an enzyme that mediates conversion of L-Arg into ornithine and urea, and is meant to encompass any or all relevant arginase types, including, for example, arginase type I, arginase type II, and the like.

[0036] "Arginase inhibitor" refers to an agent, such an organic compound or anti-arginase antibody, which agent can be either naturally-occurring or synthetic, which agent affects activity of an arginase (e.g., arginase type I, arginase type II, or both) in catalysis of L-Arg into ornithine and urea. For example, an antibody which binds arginase can affected arginase activity by interfering with arginase binding to its substrate or by promoting clearance of arginase from the subject's circulation. Production of arginase antibodies are well within the skill of the ordinary artisan, and appropriate arginase proteins for production of such antibodies are available.

[0037] As used herein, the terms "treatment," "treating," and the like, refer to obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment," as used herein, covers any treatment of a disease in a mammal,

particularly in a human, and can include: (a) preventing the disease or a symptom of a disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it (e.g., including diseases that may be associated with or caused by a primary disease); (b) inhibiting the disease or condition, i.e., arresting its development; and (c) relieving the disease, i.e., causing regression of the disease.

[0038] The terms "individual," "host," "subject," and "patient" are used interchangeably herein, and generally refer to a mammal, including, but not limited to, primates, including simians and humans., equines (e.g., horses), canines (e.g., dogs), felines, various domesticated livestock (e.g., ungulates, such as swine, pigs, goats, sheep, and the like), as well as domesticated pets and animals maintained in zoos. Treatment of humans is of particular interest.

[0039] Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0040] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0041] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0042] It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for

example, reference to "an arginine inhibitor" includes a plurality of such inhibitor compounds and reference to "the arginase" includes reference to one or more arginase polypeptides and equivalents thereof known to those skilled in the art, and so forth.

[0043] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DETAILED DESCRIPTION OF THE INVENTION

[0044] The present invention is based on the discovery that arginase plays a role in modifying L-Arg bioavailability in SCD, asthma, pulmonary hypertension, and other pathologic conditions of upregulated arginase activity. Increased arginase activity limits arginine bioavailability through its conversion of L-Arg to ornithine and urea, thereby competing with NOS for available L-Arg substrate and regulating nitric oxide (NO) production. Ornithine itself also decreases L-Arg bioavailability, since both L-Arg and ornithine compete for the same transport system for cellular uptake. Downstream by-products of arginase activity, e.g., proline and polyamines have been implicated in lung and cardiovascular pathology, by way of airway remodeling, fibrosis and vascular smooth muscle proliferation. In addition to decreasing NO bioavailability, elevated arginase activity also provides substrate for a pathway which produces metabolites that likely play a role in the pathogenesis of asthma, pulmonary hypertension and other inflammatory conditions.

[0045] There are several possible mechanisms that could lead to increased arginase activity in sickle cell disease. Chronic and acute hemolysis could result in an increased dumping of red blood cell arginase into the circulation. Long-term effects of chronic end organ damage, particularly involving the liver and kidneys, which contain high arginase concentrations, may also lead to leakage of intracellular arginase into the circulation. The inflammatory state of both sickle cell disease and asthma could play a role, as arginase gene expression is upregulated by many cytokines involved in the inflammatory process.

[0046] Without being held to theory, the present invention is based on the hypothesis that arginase plays a role in modifying L-Arg bioavailability in SCD, asthma, pulmonary hypertension, and other pathologic inflammatory conditions that upregulate arginase levels/activity. Increased arginase activity limits arginine bioavailability through its conversion of L-Arg to ornithine and urea, thereby competing with nitric oxide synthase (NOS) for

available L-Arg substrate and interfering with NO production (Figure 4). L-Arg produces nitric oxide (NO) and citrulline (cit) in the presence of the nitric oxide synthase enzyme (NOS). Nitric oxide release causes vasodilation through the activation of soluble guanylate cyclase (GTP) to the intracellular messenger cyclic GMP (cGMP). Arginase converts L-arginine to ornithine and urea. Both L-arginine and ornithine use the same Cationic Amino Acid Transporter molecule (CAT) for cellular uptake Ornithine can competitively inhibit L-arginine transport into the endothelial cell, thereby limiting substrate availability for nitric oxide synthase and regulating nitric oxide production. N^G-hydroxyl-L-arginine is the intermediate product of the L-arginine-nitric oxide pathway (33, 55), and is a potent inhibitor of arginase activity.

[0047] Accumulation of both intracellular and extracellular N^G-hydroxyl-L-arginine favors the continued conversion of L-arginine to nitric oxide by maintaining adequate arginine availability. The downstream by-products of arginase activity, i.e., proline and polyamines, likely play a role in disease pathogenesis, as they are involved in vascular smooth muscle proliferation as well as airway remodeling (Figure 4). These metabolites may accumulate in serum or plasma as seen in sickle cell patients with pulmonary hypertension. This is a novel model for the pathogenesis of pulmonary hypertension.

[0048] Proline is involved in collagen formation (44, 45) and lung fibrosis (46), processes that occur in airway wall thickening and airway remodeling (47-50). Proline plays an important function in tissue remodeling and normal wound healing (45), however overproduction can lead to pathologic states. Elevated arginase activity can lead to such conditions.

[0049] In an environment of low L-arginine concentration, nitric oxide synthase is uncoupled and reduces oxygen (O_2) to superoxide (O_2^-) instead of generating nitric oxide. Nitric oxide reacts rapidly with superoxide to form reactive nitric oxide species (RNOS) that could lead to oxidative stress and cellular damage. Pathological conditions of increased arginase activity thus would have a negative impact on nitric oxide bioavailability. In short, since both arginase and NOS use Arg as a common substrate, arginase plays a role in regulating nitric oxide (NO) synthesis by modulating L-Arg availability. Decreased arginine bioavailability leads to hyperreactive airways in both SCD and asthma, since it plays a role in bronchodilation. Thus, decreased arginine bioavailability and elevated arginase activity contributes to the disease process. Furthermore, decreased arginine bioavailability leads to pulmonary hypertension in the susceptible patient.

[0050] The data presented herein demonstrate that asthmatic patients exhibit a significant arginine deficiency during acute exacerbations that is even greater than what is observed in

patients with SCD (109.0±33.1 vs. 55.4±16.0 vs. 38.9±20μM in plasma of normal controls vs. SCD patients with pulmonary hypertension vs. asthma, respectively, $p<0.0001$, Figure 1, Panel A). Arginine levels rise significantly by discharge in asthmatics admitted to the hospital (Figure 2). In SCD, this arginine deficiency translates to decreased nitric oxide bioavailability. Arginase activity is elevated in asthmatic patients, (1.6±0.9 vs. 0.95±0.7 vs. 0.427±0.2 μmol/ml/hr, asthma vs. SCD vs. normal controls respectively, $p=0.001$, Figure 1, Panel B).

[0051] In addition, the inflammatory state of the patient's condition can also play a role, as arginase gene expression is upregulated by many cytokines involved in the inflammatory process, particularly the Th2 cytokines. Data presented herein demonstrates elevated sPLA2 levels in serum of asthmatic patients vs. normal controls (4.2±2 vs. 25.9±30, $p<0.05$, normal control vs. asthma) . Besides the basal cytokine production, the additional increase in the serum and local cytokine levels may be induced by activated lymphocytes, monocytes and other inflammatory cells.

[0052] The invention will now be described in more detail.

ARGININE AND ARGINASE INHIBITORS

Arginine

[0053] Arginine as used herein generally refers to L-arginine or "L-Arg". Arginine useful in the invention can be isolated from naturally-occurring sources, provided in an enriched source (e.g., in a foodstuff in which relatively high levels in terms of percent weight is found naturally or is modified to contain such higher levels), or produced by synthetic methods.

[0054] L-Arg can be administered as any physiologically acceptable salt, such as the hydrochloride salt, glutamate salt, nitrite, ascorbate etc . L-Arg can also be administered as a peptide (e.g., poly-L-arginine, or combinations of L-Arg and poly-L-arginine). Oligopeptides of particular interest include oligopeptides of from 2 to 30, usually 2 to 20, preferably 2 to 10 amino acids, having at least 50 mol % of L-arginine, preferably at least about 75 mol % of L-arginine, more preferably having at least about 75 mol % of L-arginine. The oligopeptides can be modified by being ligated to other compounds, which can enhance absorption from the gut, provide for enhancement of NO synthesis or stability, e.g. reducing agents and antioxidants, and the like

Arginase Inhibitors

[0055] A variety of arginase inhibitors can be adapted for use in the present invention. The arginase inhibitor can be a reversible or irreversible arginase inhibitor, or arginase antibody.

Preferably the arginase inhibitor is compatible for use, or can be adapted so as to be compatible for use, in a pharmaceutically acceptable formulation or in a nutraceutical. Exemplary arginase inhibitors include, but are not necessarily limited to, N(omega)-hydroxy-nor-L-arginine (NOHA),N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) , 2(S)-amino-6-boronohexanoic acid (ABH) (see, e.g., US Pat. No. 6,387,890), S-(+)-Amino-6-iodoacetamidohexanoic acid (irreversible); S-(+)-Amino-5-iodoacetamidopentanoic acid (irreversible); L-norvaline, L-HOArg, and the like. NOHA is of particular interest in the present invention.

Magnesium

[0056] Without being held to theory, since magnesium has a role in the L-arginine-nitric oxide pathway and attenuates endothelial dysfunction, combination therapy with arginine (with or without an arginase inhibitor) augments the brochodilatory and vasodilatory properties of magnesium through this pathway. Conditions of associated with decreased nitric oxide bioavailability (e.g., endothelial dysfunction) are amenable to treatment with arginine and magnesium (alone or with an arginase inhibitor). Such combination therapy can have synergistic benefits in treatment of conditions of decreased nitric oxide bioavailability and/or decreased arginine bioavailability.

NO

[0057] NO can be administered in a variety of forms, including, but not limited to inhalation, or as a nitric oxide (NO) donor , and the like. NO gas can be inhaled, while NO donors can be administered in a variety of ways according to the nature of the compound, the manner in which it is formulated, and the like. Exemplary NO donors include, but are not necessarily limited to hydroxyurea is an NO donor, sildenafil, nitrite, however there are many agents that are NO donors.

Formulations

[0058] L-Arg, arginase inhibitors, magnesium, or other agent for administration according to the invention (referred to herein as "the agents" for convenience) can be formulated in a variety of ways suitable for administration according to the methods of the invention. In general, these compounds are provided in the same or separate formulations in combination with a pharmaceutically acceptable excipient(s). A wide variety of pharmaceutically acceptable excipients are known in the art and need not be discussed in detail herein. Pharmaceutically acceptable excipients have been amply described in a variety of publications, including, for example, A. Gennaro (2000) "Remington: The Science and Practice of Pharmacy," 20th edition, Lippincott, Williams, & Wilkins; Pharmaceutical Dosage Forms and Drug Delivery Systems (1999) H.C. Ansel et al., eds., 7th ed., Lippincott, Williams, & Wilkins; and Handbook

of Pharmaceutical Excipients (2000) A.H. Kibbe et al., eds., 3rd ed. Amer. Pharmaceutical Assoc.

[0059] The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are readily available to the public. Moreover, pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are readily available to the public.

[0060] In some embodiments, the agents are formulated separately or in combination, e.g., in an aqueous or non-aqueous formulation, which may further include a buffer (e.g., L-Arg with an arginase inhibitor and/or magnesium, such as L-Arg with an arginase inhibitor, L-Arg with magnesium, L-Arg with both an arginase inhibitor and magnesium, for example). Suitable aqueous buffers include, but are not limited to, acetate, succinate, citrate, and phosphate buffers varying in strength from 5 mM to 100 mM. In some embodiments, the aqueous buffer includes reagents that provide for an isotonic solution. Such reagents include, but are not limited to, sodium chloride, and sugars e.g., mannitol, dextrose, sucrose, and the like. In some embodiments, the aqueous buffer further includes a non-ionic surfactant such as polysorbate 20 or 80.

[0061] Optionally the formulations may further include a preservative. Suitable preservatives include, but are not limited to, a benzyl alcohol, phenol, chlorobutanol, benzalkonium chloride, and the like. In many cases, the formulation is stored at about 4°C. Formulations may also be lyophilized, in which case they generally include cryoprotectants such as sucrose, trehalose, lactose, maltose, mannitol, and the like. Lyophilized formulations can be stored over extended periods of time, even at ambient temperatures.

[0062] In the subject methods, the agents may be administered to the host using any convenient means capable of resulting in the desired therapeutic effect. In general, administration can be by any suitable parenteral (e.g., intravenous, intramuscular, subcutaneous, and the like) or enteral (e.g., oral) route. Thus, the agents can be incorporated into a variety of formulations for therapeutic administration. More particularly, the agents of the present invention can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants and aerosols.

[0063] In pharmaceutical dosage forms, the agents may be administered in the form of their pharmaceutically acceptable salts, or they may also be used alone or in appropriate association,

as well as in combination, with other pharmaceutically active compounds. The following methods and excipients are merely exemplary and are in no way limiting.

[0064] The agents can be formulated into preparations for injection by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

[0065] For oral preparations, the agents can be used alone or in combination with appropriate additives to make tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents. In some embodiments, particularly in the case of L-Arg, the agents can be formulated in the form of a nutriceutical, e.g., as a food product, e.g., admixed with a foodstuff.

[0066] Furthermore, the agents can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. The compounds of the present invention can be administered rectally via a suppository. The suppository can include vehicles such as cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature. Agents can also be provided in sustained release or controlled release formulations, e.g., to provide for release of agent over time and in a desired amount (e.g., in an amount effective to provide for a desired therapeutic or otherwise beneficial effect).

[0067] Unit dosage forms for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit, for example, teaspoonful, tablespoonful, tablet or suppository, contains a predetermined amount of the composition containing one or more inhibitors. Similarly, unit dosage forms for injection or infusion administration may comprise the inhibitor(s) in a composition as a solution in sterile water, normal saline or another pharmaceutically acceptable carrier.

[0068] The term "unit dosage form," as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of the agents calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications

for the unit dosage forms for use in the present invention depend on the particular compound employed and the effect to be achieved, the pharmacodynamics associated with each compound in the host, and the like.

[0069] Dosage forms of particular interest include those suitable to accomplish parenteral (e.g., intravenous, intramuscular, subcutaneous, and the like) or oral administration, as well as dosage forms to provide for delivery by a nasal or pulmonary route (e.g., inhalation), e.g., through use of a metered dose inhaler and the like.

[0070] In general, arginine for use in the invention is formulated in either parenteral or enteral forms, usually enteral formulations, more particularly oral formulations. In one embodiment of particular interest, L-Arg is administered in the form of a dietary supplement, which can be provided as, for example, a drink, powdered drink or foodbar. Where the subject has asthma, administration of the agent (e.g., arginine) in an inhaled formulation that is free of irritants, or by a route other than inhalation (e.g., oral or by injection), may be preferred.

[0071] Arginase inhibitors for use in the invention are formulated for parenteral administration, e.g., by subcutaneous, intradermal, intraperitoneal, intravenous, or intramuscular injection. Administration may also be accomplished by, for example, enteral, oral, buccal, rectal, transdermal, intratracheal, inhalation (see, e.g., U.S. Pat. No. 5,354,934), etc.

[0072] Arginine and arginase inhibitors may be administered as separate dosage forms by the same or different route, or may be formulated as a single dosage form. In one embodiment, arginine and an arginase inhibitor are administered in the form of a capsule, foodbar, or drink, where the two agents may be in separate dosage forms or combined in the same dosage form. In another embodiment, arginine and an arginase inhibitor are provided in the same or different formulation for nebulized delivery. Nebulized delivery may be of particular interest for administration for treatment of asthma and pulmonary hypertension.

[0073] Magnesium is generally be administered as a pharmaceutically acceptable magnesium salt, such as, for example, magnesium sulfate, magnesium chloride or the like. Magnesium can be administered as an oral preparation or medicinal food, an intravenous preparation, and/or it can be nebulized as an inhalant. Exemplary dosing for nebulization includes but is not limited to at least about 3cc (3.2 % soln, 95mg), which can be administered as a one-time dose, a continuous nebulization over one to several hours, or every 5minutes, 10 minutes, 15 minutes, 20 minutes, 30 minutes, hourly, or other dosing schedule as may be medically indicated (e.g., by a clinical practitioner). Exemplary intravenous dosing includes, but is not limited to, at least about 10 mg/kg to about 500 mg/kg, with exemplary and oral dosing of, for example, at least

about 200 gm/day to about 1000 gm/day given as a single dose or divided BID, TID or QID as medically indicated may be used.

Additional agents for combination therapy

[0074] In addition to combination therapy involving administration of L-Arg alone and/or with and an arginase inhibitor, the invention also contemplates administration of additional agents. In one embodiment of particular interest, nitric oxide (NO) donors, and/or NO in the form of inhaled NO gas, is administered to the subject. In the context of treatment of asthma, the therapeutic methods of the invention can further include administration of magnesium and/or anti-inflammatory agents such as, for example, phospholipase inhibitors, particularly cytosolic or secretory phospholipase (PLA, e.g., phospholipaseA2 (PLA2)), leukotriene inhibitors, corticosteroids.

[0075] Additionally, patients with asthma as well as those with sickle cell disease demonstrate deficiencies in many amino acids. Since extracellular arginine deprivation has been shown to influence intracellular amino acid concentrations, improved arginine bioavailability can serve to normalize some of the aberrant amino acid patterns seen in these disease states. However, combination therapy of other deficient amino acids, such as those indicated as deficient in the examples below, in addition to an agent described herein (e.g., arginine and/or an arginase inhibitor and/or magnesium) can also be beneficial and is included in this invention. Exemplary PLA inhibitors that may be useful are described in U.S. Pat. Nos. 6,492,550; 6,443,001; 6,214,876; 5,641,800; and 5,514,704.

[0076] It is well within the skill of the ordinary artisan, given the guidance provided herein, to select a dose and dosage regimen of L-Arg, and/or an arginase inhibitor and/or magnesium to provide for a desired therapeutic or otherwise beneficial effect in the subject. Precise doses and dosage regimens can vary with such factors as, for example, whether L-Arg is administered as a monotherapy or in combination with an arginase inhibitor and/or magnesium and/or other agent, subject-dependent factors (e.g., body metrics (e.g., weight, height, size, body surface area, and the like), health, tolerance to agent and/or formulation, and the like); agent-dependent factors (e.g., pharmacokinetics (e.g., including serum half-life), bioavailability, and the like); dosage regimen-dependent factors (e.g., route of administration, course of therapy, and the like); and dosage form-dependent factors (e.g., formulation, bolus dosage form, sustained release dosage form, and the like). In general, Arg is administered in a dose of are up to 0.1 g/kg body weight BID (twice daily) to TID (three times daily) with a maximum dose of about 30 gms/day. Lower doses can be administered where arginase inhibitor provides for increased

arginine bioavailability, as discussed above. Doses of arginase inhibitor can readily be determined, and in generally are lower amounts than that for arginine.

METHODS OF TREATMENT AND SUBJECTS AMENABLE TO TREATMENT ACCORDING TO THE INVENTION

[0077] Any subject having a condition associated with decreased nitric oxide bioavailability, such as that which results from decreased arginine bioavailability, elevated arginase (e.g., arginase activity and/or arginase levels), or decreased NO bioavailability are amenable to therapy according to the invention. Such therapies include administration of L-Arg (e.g., as a dietary supplement, etc.), which in embodiments of particular interest is administered in conjunction with an arginase inhibitor (e.g., NOHA, arginase antibodies), magnesium, or combinations thereof. For example, magnesium can be administered in conjunction with L-Arg or in addition to a combination therapy of L-Arg and arginase inhibitor. Optionally, NO (e.g., in the form of an inhaled gas or NO donor) can be administered in conjunction with L-Arg monotherapy or combination therapy of L-Arg and arginase inhibitor and/or L-Arg and magnesium. The phrase "in conjunction with" means that an agent is administered prior to, concurrently, or after other substance or therapy.

[0078] The agents (e.g., L-Arg, arginase inhibitor, magnesium, NO) can be administered as separate formulations or, where feasible, as a combined formulation. The agents can be administered at the same time or at different times. Dosages of agents in each of the contexts above can be based upon the various factors as described above. In general, doses may be administered TID (three times a day), BID (twice a day) or QID (four times daily)or QD (daily). For example, the particular regimen for arginase inhibitor (and for arginine) will vary according to a variety of patient factors. For example, where the patient to be treated has sickle cell disease, TID or BID may be of particular interest. For status asthmaticus, therapy may be administered as a one-time dose in the acute setting, or QD, BID, TID, or QID as deemed medially appropriate.

[0079] Exemplary conditions associated with decreased nitric oxide bioavailability and/or elevated arginase levels (relative to non-disease individuals) include, but are not necessarily limited to asthma, sickle cell disease (SCD), pulmonary hypertension (in SCD, neonatal pulmonary hypertension and/or persistent pulmonary hypertension of the newborn, primary hypertension, secondary hypertension), pneumonia, chronic obstructive pulmonary disease (COPD), systemic hypertension, pregnancy related hypertension (pre-eclampsia/eclampsia, arteriosclerosis, diabetes, trauma injury, sepsis, cystic fibrosis, erectile dysfunction, and

hemolytic disorders (where the source of elevated arginase activity is via release from the red blood cell). Conditions amenable to therapy include those that have been previously treated (e.g., as in steroid therapy for asthmatics) or that have not been previously treated ("treatment naïve").

[0080] By "elevated arginase levels" is meant that the subject exhibits a level of arginase activity that is about 20% greater, usually more than about 20% greater, than arginase activity of an average normal subject. Arginase activity measurements in serum or plasma are a special test that is not routinely available. Specialized laboratories can provide this service. Results may vary depending upon the laboratory performing the analysis. Therefore, results must be compared to normal controls (i.e., patients without an inflammatory condition that might be associated with increased arginase activity). Normal, unaffected humans (as reported by Waugh et al, Nutritional Research. 1999. 19;501-518) demonstrate plasma arginase activity levels of 0.2±0.3μM/ml/30 min. The present inventor has observed normal serum arginase activity of 0.4±0.2μM/ml/hr. Thus, arginase activity in plasma and serum of normal controls are low. Levels that are at least about 20% or more above normal are considered elevated. For example, a serum arginase level that is ≥0.6μM/ml/hr would generally be considered an elevated arginase level.

[0081] Asthma is a complex syndrome with many clinical phenotypes that involve a multitude of mechanisms, influenced also by genetic and environmental factors. Individual patient response to asthma therapy also varies, and is likely a reflection of the various mechanisms responsible for disease development and severity. The invention is indicated for those types of asthma that involve elevated arginase activity, decreased arginine bioavailability, and/or limited nitric oxide bioavailability. Included in this group are all varieties of asthma (e.g., allergic asthma, nocturnal asthma, exercise-induced asthma, mild-intermittent, moderate intermittent, moderate persistent, severe persistent, etc). The same is true for the various forms of pulmonary hypertension, and other diseases that manifest with similar clinical symptoms or phenotype but possess underlying mechanistic differences. Altered arginine and nitric oxide bioavailability are likely a common denominator in many of these disease processes, and as such, are amendable to treatment described in this invention.

[0082] This invention may be utilized for acute care during exacerbations of the above described conditions, for treatment of the chronic condition, and/or as prophylaxis to avoid development or progression of the described conditions. Many of these conditions have genetic modifiers that have already been identified that put an individual at risk for developing

certain diseases, and such techniques (including but not limited to HLA testing, microarray analyses, evaluation of genomic polymorphisms etc) may be helpful in identifying patients who would benefit from this invention.

Arginase levels

[0083] Arginase levels and/or arginase activity can be assessed according to methods well known in the art. See, e.g., Morris et al. Am J Physiol Endocrinol Metab 1998;275:740-747. For example, arginase levels can be assessed in blood (e.g., whole blood or serum, plasma, or other blood fraction), bronchoalveolar lavage, or in target organ tissue samples (e.g., found on biopsy). As used herein "detection of arginase" is meant to encompass detection of arginase protein in a sample, detection of activity of arginase in a sample, or both.

[0084] Arginase activity, particularly that present in the serum or plasma of patients, may also be assessed based on the arginine-to-ornithine ratio. This ratio is also helpful in evaluating arginine bioavailability, which is limited by elevated plasma ornithine levels through competitive inhibition of cellular uptake of arginine. The present inventor has found that the arginine/ornithine ratio is significantly lower in sickle cell patient with pulmonary disease (pulmonary hypertension). Likewise, the present inventor has found that the arginine/ornithine ratio is significantly lower in asthmatics, compared to normal controls (0.94 ± 0.5, $n=26$ vs. 1.6 ± 0.6, $n=15$, $p=0.003$).

[0085] In normal control patients studied, arginine levels were usually greater than ornithine levels, such that the ratio often approached 2:1. Such a ratio would avoid a limitation on arginine bioavailability purely on the basis of competitive inhibition, since Arg and ornithine share the same amino acid transporter molecules. Without being held to theory, as the ornithine concentration rises, and the arginine-to-ornithine ratio decreases, arginine bioavailability becomes limited even under conditions of apparently normal arginine concentration. Pathologically elevated arginase activity reduces the arginine-to-ornithine ratio by utilizing arginine (and decreasing that which is available to nitric oxide synthase to make nitric oxide), while hydrolyzing arginine to ornithine, the substrate for proline and polyamine production, metabolites likely involved in disease pathogenesis

[0086] A low arginine-to-ornithine ratio, thus, is a reflection of increased arginase activity. Once this ratio nears or drops below 1, arginine availability for nitric oxide production has reached a competitive disadvantage. An arginine-to-ornithine ratio of less than about 1.2 is considered low. Patients with such a finding, regardless of the disease pathology, may be

treated with the arginine/arginase inhibitor combination therapy, arginine/magnesium combination therapy, or other therapy of the invention.

Assessing therapy

[0087] Following administration of a therapy according to the invention, efficacy can be assess in the patient by, for example, observing an improvement or stabilization in one or more symptoms relevant to the disease being treated. Therapy can also be assessed by assessing arginase levels or activity and/or a normalization of the arginine-to-ornithine ratio. Doses of agents administered can be adjusted in accordance to patient need, e.g., to provide for a decrease of arginase activity levels to within a normal range, e.g., within a range such that arginase levels are not above normal levels more than about 5%, 10%, 15%, or 20%, or a sufficient increase in plasma arginine concentration to the extent that arginine bioavailability is no longer limiting factor for nitric oxide production, ie levels above the Km for arginine transport (>120μM), and a normalization of the arginine-to-ornithine ratio (>1.5).

[0088] Therapy can be assessed by examining improvement in one or more clinical symptoms of disease. Successful therapy is normally considered to be a significant improvement in one or more clinical symptoms after treatment according to the invention as compared to prior to such treatment. In some embodiments, an "effective amount" of L-Arg, or an effective amounts in the context of a combination of L-Arg and an arginase inhibitor, is a dosage that is effective to improve one or more clinical parameters of the condition by at least about 10%, at least about 15%, at least about 25%, at least about 50%, or more, compared to the clinical parameter prior to therapy, or compared with a placebo control or an untreated control. For example, in pulmonary hypertension, clinical parameters assessed can be one or more of: an improvement in mean pulmonary artery systolic pressure as estimated by tricuspid regurgitant jet velocity measured by Doppler-echocardiograpy, improved exercise tolerance as measured by a "6-minute walk"; blood pressure in systemic hypertension, etc),.

[0089] In the context of conditions that affect lung function, the clinical parameters can be, for example, forced inspiratory flow (FIF), forced expiratory flow (FEF), forced vital capacity (FVC), diffusing capacity for carbon monoxide (DLco), and/or the like. For example, in asthma, therapy can be assessed by spirometry, lung volume, airway resistance, and/or oxygen saturation. In patients having pulmonary hypertension, therapy can be assessed using lung function tests, as well as assessing mean pulmonary artery pressure (e.g., at rest and/or with exercise). It should be noted that successful therapy according to the invention includes outcomes where the underlying disease state is not significantly altered, but one or more

clinical symptoms (including symptoms that arise from or are associated with the disease) are treated.

[0090] In the context of sickle cell disease, clinical parameters include, for example one or more of: a decrease in the number of pain crisis, number emergency department visits, number of hospitalizations and/or duration of hospitalization, amount of pain medication use, incidence of and/or occurrence of complications such as skin ulcers, need for transfusion, oxygen use, etc. Also improved pain scores and quality of life assessment tools can be followed.

KITS

[0091] Kits with unit doses of L-Arg formulation, an arginase inhibitor formulation, and/or magnesium formulation (which formulations may be combined or separate as described herein) suitable for use in the methods of the invention are provided. In such kits, in addition to the containers containing the unit doses will be an informational package insert describing the use and attendant benefits of the agents in treating conditions associated with elevated serum arginase activity

[0092] In some embodiments, a subject kit includes a container comprising a formulation comprising a unit dose of L-Arg, an arginase inhibitor, magnesium, or combination thereof, and a pharmaceutically acceptable excipient; and instructions to administer the dosage form according to a desired regimen or exemplary regimen dependent upon the particular condition to be treated, patient age, patient weight, and the like. The instructions can be printed on a label affixed to the container, or can be a package insert that accompanies the container.

[0093] In another embodiment, the agents for administration (e.g., L-Arg, arginase inhibitor, magnesium, NO) are provided in the kit along with materials to facilitate analysis of serum arginase levels in the subject who is a candidate for therapy according to the invention.

EXAMPLES

[0094] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric.

METHODS AND MATERIALS

[0095] The following methods, materials, and patient populations relate to those referred to in the Examples below.

[0096] ***Asthma patients.*** Patients with asthma presenting to the emergency department and clinics at Children's Hospital and Research Center at Oakland were recruited. Blood samples and exhaled nitric oxide levels (in patients old enough to perform peak flow) are obtained at presentation to the emergency department or clinic, and followed daily during hospitalization for those patients ill enough to require admission.

[0097] Baseline blood was obtained at least 4 weeks after resolution of the acute exacerbation. Blood samples were analyzed for arginine and amino acid levels, arginase activity, and arginine-to-ornithine ratio. Additional analyses that may be performed include analysis of TH-2 cytokines cytokines, VCAM and ICAM, nitric oxide metabolite levels (in blood, breath and urine), genetic markers, IgE, Pla2 levels, RSV (in < 2 year old acutely wheezing) and proteomic analysis. A clinical asthma score routinely used at Children's Hospital Oakland, peak flows (when age appropriate) is obtained, and a symptoms questionnaire (see appendix) is filled out on each patient.

[0098] Well asthmatics (mild intermittent under good control) and non-asthmatic normal controls will also be recruited for comparison. Wheezing infants who do not carry the diagnosis of asthma will also be recruited for participation in this study in order to determine whether elevated arginase, Th2 cytokines and genetic modifiers can differentiate a subgroup of patients likely to develop asthma (as defined by 3 or greater episodes of wheezing). Follow-up phone calls to these families are done in order to determine repeat episodes of wheezing 1 year after enrollment. A paired student *t*-test and ANOVA is used for repeated measurements within the same patient, and an unpaired student *t*-test is used to compare different groups.

[0099] ***Sickle cell patients.*** Seventeen sickle cell disease patients with documented pulmonary hypertension at steady-state were enrolled in the study. All known patients with pulmonary hypertension receiving care at the Northern California Comprehensive Sickle Cell Center were approached for participation in this analysis. Twelve patients were homozygous for hemoglobin S, three patients had hemoglobin type SC, and two patient had hemoglobin S β-thalassemia. The mean age of patients was 32.7±15 years with a range of 13 to 63 years. There were seven women enrolled. Ten ethnically matched normal non-sickle cell disease volunteers were enrolled as a control group in order to compare amino acid levels and arginase activity. The mean age was 20.6±10 years, ranging from 10 to 34 years. There were four females and

six males enrolled. Pulmonary hypertension was defined as estimated pulmonary artery pressures > 30 mm Hg by echocardiogram (or tricuspid regurgitant jet velocity of greater than 2.5m/sec), > two months duration, not associated with acute chest syndrome. A chart review was performed on all patients to obtain tricuspid regurgitant jet velocity data from previous echocardiograms.

[00100] ***Amino Acid Levels.*** (A complete amino acid panel, including arginine, citrulline, ornithine, and L-arginine analogue asymmetric di-methyl-L-arginine). Quantitative plasma amino acid levels are measured in μmol/L, using a Beckman 6300 amino acid analyzer. The amino acids are separated on an lithium ion exchange column and then reacted with ninhydrin to generate a color response. The data is collected and analyzed using Beckman 32 Karat software, at the Molecular Structure Facility, University of California, Davis, CA.

[00101] ***Arginase***: Arginase-specific activity is determined in plasma by methods previously described. (36)

[00102] ***NOAnalyzer***: Serum is stored at -70° until assayed for nitrate/nitrite/S-NO. NO_x can be measured in serum, plasma or urine according to manufacturer's instructions, using Sievers NOAnalysis software for liquid sampling (Sievers Instruments, Inc., Denver, CO), as previously described.(38-40) Briefly, serum nitrite is measured by acidifying serum to a pH <2.0 to convert nitrite to NO. Serum nitrate is measured by incubating serum with Aspergillus nitrate reductase (Boehringer, Mannheim) to reduce nitrate into nitrite and then convert nitrite into NO by the addition of hydrochloric acid. The NO produced is then injected into the NO analyzer (Sievers, Inc), and the NO content of the sample is determined by measuring the luminescence generated in the presence of ozone. The luminescence measured is directly proportional to the amount of NO injected and, in turn, to the nitrite and nitrate content of the samples. Serum samples can be run immediately, or frozen for later analysis.

[00103] ***Exhaled Nitric Oxide***: Exhaled nitric oxide is measured in exhaled air, using microprocessor-based chemiluminescent NO_x analytical instrumentation, manufactured by Sievers Instruments, Inc. (Denver, CO). The test is easily performed and has been successfully used in many clinical trials. (12, 41, 42) Subjects inhale to total lung capacity from a reservoir bag through a one-way valve (Hans Rudolph, Kansas City, MO) with incoming NO-free air to ensure the absence of environmental NO. Next, the subjects exhale to residual volume into the Teflon tube, which enters into the NO analyzer. The subjects exhale at a pressure of +20 mmHg into the tubing connected to the analyzer. Exhalation at this expiratory pressure without

a nose clip is a maneuver that closes the velum of the posterior nasopharynx and excludes contamination by nasal NO.

[00104] ***Immunofluorescence staining and flow cytometry (FACS) analysis.*** Whole blood samples collected into preservative free heparin is used. Monoclonal antibodies used for staining are: FITC conjugated CD3, CD25, CD69, CD80, CD86, CD95 (Immunotech, Westbrook, ME), PE conjugated CD 154 (CD40L), CD16, CD56, CD63 (Becton Dickinson, San Jose, CA), FITC conjugated CD45RA, CD40 (Coulter, Hialeah, FL), PE conjugated CD45RO (Beckton-Dickinson, CA), PerCP conjugated CD3, CD4, CD8, CD19 (Beckton-Dickinson, San Jose, CA). Two- and three-color analyses are performed on the FACScan (BDIS, Mountain View, CA). 10,000 events are acquired and analyzed.

[00105] ***T cell activation.*** Heparinized blood is diluted 1:1 with RPMI and incubated for 8 hours at 37 C with or without the presence of 10ng/ml of PMA and 1microg/ml of ionomycin (Sigma Chemical Co.)

[00106] ***Mitogen and antigen blastogenesis.*** Blood mononuclear cells are stimulated with mitogens or specific antigens to undergo cell division and proliferation. This process is monitored by measurement of thymidine incorporated into newly synthesized DNA within the cells. The mitogen which is used is Phytohaemagglutinin (PHA)(Difco, Detroit), in the working dilutions 1:25, 1:125, 1:625. Antigens will consist of Tetanus Toxoid (Connaught Laboratories Limited, Willowdale, Ontario), Candida (Miles Inc.), cytomegalovirus (CMV), herpes simplex virus (HSV), and varicella-zoster virus (VZV)(Myron J. Levin, M.D. UCHSC, Denver.CO). All reactions are run in triplicate with 10^5 cells plated per well. Incubation time for mitogen assays is 3 days and while that for antigen is 7 days, both at 37°C in 5% CO_2 . The cells are pulsed on the last day by adding 50ul of ^{3}H-Thymidine to each well for a final concentration of 1 uCi/well. The plates are harvested 6 to 18 hours after pulsing.

[00107] ***sPLA2:*** sPLA2 protein is measured using ELISA and sPLA2 activity using breakdown of thioester via methods previously described (61).

[00108] ***Serum levels of cytokines*** . We will use frozen serum samples to measure *TNF a, sIL-2R, IL-1, IL-2, IL-4, IL-6, IL-10, g-Interferon and CD40L* . A commercially available ELISA kit for cytokines is routinely used, according to the manufacturer' instructions (R&D Systems, Minneapolis and Immunotech, Westbrook, ME). ELISA kits for VCAM, ICAM and levels of sCD40L have recently become available from Chemicon, CA.

[00109] ***Genetic Markers.*** NO is synthesized in endothelial cells from L-arginine by the enzyme nitric oxide synthase (NOS) and there are known single nucleotide polymorphisms (SNPs) in the NOS3 gene. Since NO may play a key role in the regulation of bronchomotor

tone and inflammation of the airways (62), genetic studies evaluating the NOS gene in asthmatics may would be of interest. A method for rapidly genotyping multiple SNPs simultaneously has been developed at Roche Molecular Systems, Alameda, CA. An example of multiplex PCR products is shown in the agarose gel below. These 18 PCR products contain SNPs in genes thought to play a role in asthma: TNFα; CCqα; TNFR1: TNFβ; IL5Rα; TNFβ; IL9; CCR2; IL4Rα; CCR5: RMS1; β2AR; CC16; FcεRIβ; CTLA4; SCYA11; IL4Rα; IL4; and IL6.

EXAMPLE 1: ANALYSIS OF AMINO ACID LEVELS IN ASTHMATICS, SICKLE CELL DISEASE, AND PHT PATIENTS

[00110] Reductions were seen in plasma levels of many amino acids in asthmatic patient experiencing an acute exacerbation of respiratory symptoms (Table 1). Strikingly, the greatest decrease was in plasma levels of arginine, which were approximately half those of normal controls (45 ± 22 μM vs. 94 ± 29 μM; $p < 0.0001$).

Table 1. Plasma Amino Acids in Normal Controls vs. Asthma

Amino Acid	Concentration (μM)		% Control	p-value
	Controls (n = 15)	Asthma (n = 26)		
Arginine	94 ± 29	45 ± 22	48	< 0.0001
Ornithine	64 ± 21	49 ± 24	77	NS
Citrulline	30 ± 6	21 ± 10	70	0.002
Proline	195 ± 66	144 ± 73	74	0.03
Hydroxyproline	29 ± 14	19 ± 9	66	0.02
Lysine	162 ± 33	112 ± 57	69	0.004
Glutamic Acid	55 ± 29	40 ± 16	73	0.04
Glutamine	554 ± 86	466 ± 148	84	0.04
Glycine	251 ± 64	186 ± 103	74	0.03
Alanine	369 ± 104	292 ± 96	79	0.02
Valine	223 ± 52	161 ± 51	72	<0.001
Aspartic Acid	9 ± 6	7 ± 1	78	0.04
Threonine	136 ± 29	99 ± 58	73	0.02
Isoleucine	66± 20	48 ± 23	73	0.01
Leucine	126 ± 32	96 ± 45	76	0.03
Tyrosine	72 ± 15	52 ± 20	72	0.002
Histidine	75 ± 10	57 ± 20	79	0.003
Cysteine	22 ± 13	20 ± 16	90	NS
Asparagine	35 ± 15	41 ± 18 (n = 25)	118	NS
Serine	107 ± 32	89 ± 64	83	NS
Tryptophan	45 ± 10	37 ± 15	82	NS
Methionine	25 ± 6	20 ± 13	80	NS

Amino Acid	Concentration (μM)		% Control	p-value
	Controls (n = 15)	Asthma (n = 26)		
Phenylalanine	57 ± 13	56 ± 17	98	NS

Concentrations of amino acids are expressed as means ± SD. % Control values reflect percentages of controls for the asthma group.

[00111] As arginine, ornithine and lysine are taken up by cells via the same y+ transport system, the ratio arginine/(ornithine + lysine) provides an index of relative arginine availability at any given plasma arginine concentration. Relative arginine availability also was significantly lower in asthmatic patients as compared to normal controls (0.30 ± 0.13 vs. 0.42 ± 0.14, $p < 0.005$), further limiting arginine availability in the asthma group.

[00112] Plasma levels of ornithine (Table 1), a product of arginine catabolism, were generally lower in asthmatics relative to controls, and relative ornithine availability (ornithine/(arginine + lysine)) was somewhat higher in asthmatics than in controls (0.25 ± 0.07 for controls, 0.34 ± 0.17 for asthma), but neither of these trends reached statistical significance. On the other hand, citrulline, the precursor of endogenous arginine synthesis, was significantly reduced in asthmatics relative to normal controls (Table 1), possibly contributing to the decrease in plasma arginine levels in these patients.

[00113] Table 2 shows plasma amino acids in normal controls vs. patients with sickle cell disease (SCD). An abnormal amino acid profile is found in patients with sickle cell disease. The greatest deficiency is found in plasma arginine concentration.

Table 2: Plasma Amino Acids in Normal Controls vs. SCD

Amino Acid	Concentration (μM)		% Control	*p-value*
	Controls (n = 29)	*SCD (n = 163)*		
Nonessential:				
Arginine	**65 ± 16**	**40 ± 15**	**62**	**<0.0001**
***Ornithine**	61 ± 22	64 ± 23	--	NS
***Citrulline**	27 ± 11	25 ± 14	--	NS
***Proline**	**141 ± 49**	**205 ± 76**	**145**	**<0.0001**
***Glutamic acid**	**38 ± 15**	**47 ± 24**	**124**	**0.04**
Glutamine	**515 ± 129**	**607 ± 125**	**118**	**0.0004**
Glycine	**205 ± 48**	**278 ± 98**	**136**	**0.0001**
Tyrosine	**61 ± 13**	**53 ± 19**	**87**	**0.03**
Alanine	330 ± 69	321 ± 110	--	NS
***Cysteine**	40 ± 7	45 ± 15	--	NS
Serine	93 ± 15	94 ± 23	--	NS
Asparagine	44 ± 13	43 ± 14	--	NS
Essential:				
Lysine	**161 ± 30**	**143 ± 34**	**89**	**0.006**

Histidine	73 ± 15	56 ± 16	77	<0.0001
Phenylalanine	61 ± 13	53 ± 19	87	0.03
***Leucine**	114 ± 25	89 ± 28	78	<0.0001
***Valine**	207 ± 41	162 ± 45	78	<0.0001
Isoleucine	58 ± 13	49 ± 16	84	0.008
Methionine	25 ± 5	26 ± 7	--	NS
Threonine	137 ± 31	126 ± 45	--	NS

Concentrations of amino acids are expressed as means ± SD.

% Control: Values are shown only when significantly different from controls.

*Amino acids that are altered in SCD patients with PHT vs. SCD patients without PHT

[00114] Table 3 illustrates plasma amino acid levels that differ in sickle cell disease patients with pulmonary hypertension compared to those without pulmonary hypertension. Elevated downstream by-products of arginase activity occur in SCD patients who have developed pulmonary hypertension.

Table 3: Plasma Amino Acids in SCD with PHT vs. SCD with PHT

Amino Acid	Concentration (μM)			*p-value*
	Controls (n=29)	*TR jet < 2.5 (n=86)*	*TR jet ≥ 2.5 (n=41)*	*(PHT vs non PHT)*
Nonessential:				
Ornithine	61 ± 22	59 ± 20	69 ± 23	0.02 (↑)
Citrulline	27 ± 11	*22 ± 10	29 ± 20	0.008 (↑)
Proline	141 ± 49	*192 ± 74	*236 ± 87	0.003 (↑)
Glutamic acid	38 ± 15	*45 ± 16	*60 ± 37	0.003 (↑)
Cysteine	40 ± 7	43 ± 14	*48 ± 16	0.04 (↑)
Essential:				
Valine	207 ± 41	*165 ± 41	*145 ± 48	0.01 (↓)
Leucine	114 ± 25	*92 ± 25	*78 ± 30	0.006 (↓)

Concentrations of amino acids are expressed as means ± SD.

*Amino acids that differ significantly ($p<0.05$) from controls

EXAMPLE 2: ARGININE AND ARGINASE LEVELS IN ASTHMATIC PATIENTS AND SICKLE CELL DISEASE (SCD) PATIENTS WITH PULMONARY HYPERTENSION

[00115] SCD and asthmatic patients exhibited a significant arginine deficiency during acute exacerbations. Serum arginine levels are summarized in the table below, and presented in Figure 1 (Panel A).

	Normal	SCD with PHT	Asthma
Serum arginine (μM)	109.0±33.1	55.4±16.0	38.9±20

PHT = pulmonary hypertension; $p<0.0001$ for comparison of SCD with PHT vs. normal, and for asthma vs. normal.

[00116] Arginase activity was elevated in SCD patients with PHT relative to normal controls, and was even greater in asthmatic patients. Serum arginase activity levels are summarized in the table below, and the data presented in Figure 1 (Panel B).

	Normal	SCD with PHT	Asthma
Serum arginase activity (μmol/ml/hr)	0.427±0.2	0.95±0.7	1.6±0.9

$p=0.001$ for comparison of SCD with PHT vs. normal, and for asthma vs. normal.

[00117] Figure 1 (Panel B) is a graph showing arginase activity in normal non-asthmatic controls (Normal, n=10) vs. patients with sickle cell disease and pulmonary hypertension (SCD, n=17) vs. patients with asthma (Asthma, n=20). Arginase activity was significantly increased in patients with asthma compared to normal controls ($p<0.001$). Arginase activity is even higher in asthmatics compared than sickle cell patients with pulmonary hypertension. Two patients with SCD having the highest levels of arginase activity died within 1 year of obtained values. Elevated arginase activity may be a reflection of increased disease severity in sickle cell disease, and is likely an inflammatory marker in asthma that potentially plays a role in disease pathogenesis.

[00118] As illustrated in Figure 2, arginine levels rose significantly by discharge in asthmatics admitted to the hospital ((54.7±29 vs. 93.1±37 μM, $p<0.05$, n = 4). Serial arginase activity levels were available on two patients and dropped substantially by discharge in each case (1.85 decreased to 1.12 μmol/ml/hr and 3.86 decreased to 0.50 μmol/ml/hr). It is likely that high arginase activity in asthmatic patients contributes to low circulating arginine levels, thereby limiting arginine bioavailability and creating a nitric oxide deficiency that induces hyperreactive airways.

[00119] Figure 3 represents changes in plasma arginine and ornithine concentration, arginase activity and nitric oxide metabolites during hospitalization in a representation four-year old boy with status asthmaticus. Sequential plasma arginine (*filled circles*) and ornithine levels (*unfilled circles*) are followed over a three-day hospitalization. Day "*1*" is the day of admission, obtained in the emergency department, and day "*3*" is the day of discharge. As shown in Panel A of Figure 3, low arginine levels increase significantly during the course of hospitalization, as does the arginine-to-ornithine ratio (0.65, day 1 vs. 1.6, day 2 vs. 1.9, day 3).

[00120] As shown in Panel B of Figure 3, serum nitric oxide metabolites (*unfilled circles*) and arginase activity (*filled circles*) are also followed over the three-day hospitalization. Arginase activity dropped dramatically as the patient clinically improved, and reached a normal level by discharge, corresponding to an increase in serum nitric oxide metabolite production. An improvement in arginine and nitric oxide bioavailability occurred as the asthma exacerbation resolves.

[00121] In addition, the inflammatory state of the patient's condition can also play a role, as arginase gene expression is upregulated by many cytokines involved in the inflammatory process, particularly the Th2 cytokines. Elevated sPLA2 levels were observed in asthmatic patients vs. normal controls (4.2±2 vs. 25.9±30, $p<0.05$, normal control vs. asthma) in serum. Since phospholipase A2 is a precursor to leukotrienes, elevated sPLA2 may identify patients who would respond to leukotriene inhibitors. Combination therapy of one or more agents described herein with leukotriene inhibitors or sPLA2 inhibitors/antibodies is beneficial for patients with asthma and other inflammatory conditions involving elevated cytokines.

EXAMPLE 3: ARGINASE LEVELS OF SCD PATIENTS WITH PHT AFTER TREATMENT WITH ARGININE

[00122] Patients with sickle cell disease and documented pulmonary hypertension by echocardiography were treated with oral L-arginine-HCl, at a dose of 0.1g/kg TID for five days. Echocardiograms were performed before and after L-arginine administration, on Day 0 and Day 6, and at ≥ 1one month follow-up after completion of arginine therapy. Blood samples for determination of amino acid levels were drawn in the morning of Day 0 (pre-treatment), Day three3, and Day six of the study. Arginase activity levels were determined on Day 0. No patients were being concurrently treated with vasodilators or anticoagulant agents, and no patients received a red blood cell transfusion during the five-day study period. Cardiologists involved in the interpretation of echocardiograms were unaware of the therapy given.

[00123] *Echocardiography.* Oral L-arginine supplementation significantly reduced pulmonary artery systolic pressure by a mean of 15.2% (63.9±13 to 54.2±12 mmHg, $p=0.002$) after five days of therapy. One patient was determined to be non-compliant based on plasma L-arginine concentration at the end of the study (61.5 μM/L at Day 0 vs. 44.9μM/L Day 6). He was the only patient found to not show an improvement in pulmonary hypertension by echocardiogram.

[00124] The tricuspid regurgitant jet velocity from echocardiograms obtained > two months prior to study enrollment demonstrated stable estimated pulmonary artery systolic pressures in five5 patients, and worsening pulmonary hypertension in two patients. Results were unavailable from outside hospitals in three patients. Follow-up echocardiography was obtained at ≥ one month after arginine therapy in the nine compliant patients, with mixed results. The non-compliant patient was lost to follow-up. Four patients reverted to their previous baseline pulmonary artery systolic pressures, four patients exhibited persistent improvement, and one patient demonstrated a worsening of pulmonary hypertension (echocardiography done while admitted for acute chest syndrome). Two of the patients demonstrating persistent improvement had also been started on transfusion therapy due to the severity of their disease, and one of these two patients had continued arginine therapy (at a dose of 0.1 gm/kg BID).

[00125] *Amino Acid levels.* Plasma L-arginine levels were low in patients with pulmonary hypertension compared to normal controls (50.8±19 vs. 114±27μM, $p<0.0001$), but similar to levels found in sickle cell patients at steady-state who did not have pulmonary hypertension. However the arginine-to-ornithine ratio was significantly lower in patients with pulmonary hypertension compared to normal controls (0.95±0.3 vs 2.0±0.6, $p<0.0001$), suggesting increased arginase activity and decreased arginine bioavailability. Both L-arginine and ornithine concentrations increased significantly after five days of oral L-arginine supplementation ($n=10$, $p<0.05$).

[00126] *Arginase activity.* Arginase converts L-arginine to ornithine and urea. Arginase activity in serum was higher in sickle cell patients with pulmonary hypertension compared to normal controls (0.82±0.6 vs. 0.43±0.2μmol/ml/h). Of interest, the patients with the two highest levels of arginase activity (1.22 and 2.46μmol/ml/h) have died within one year of enrollment. Elevated arginase activity may be a marker for disease severity.

EXAMPLE 4: L-ARG AND NOHA COMBINATION THERAPY

[00127] The effect of L-Arg and the arginase inhibitor NOHA, alone and in combination in the treatment of SCD is examined. The effect agents are examined on cell sickling, red cell indices, on functional properties of hemoglobin and on the existence of adverse effects such as hemoglobin oxidation and red cell hemolysis. The effect of the agents on interactions between sickle cells and endothelial cells, membrane transport properties and cell volume control are also examined. *In vivo* studies are performed using various lines of transgenic mice which

produce different levels of Hemoglobin S, including those which produce human Hb S/Hb F exclusively.

EXAMPLE 5: ARGININE MONOTHERAPY AND COMBINATION THERAPY OF ARGININE AND MAGNESIUM

[00128] A randomized, double-blinded placebo-control trial of intravenous arginine or arginine and magnesium for the treatment of status asthmaticus is conducted as follows. Patients with respiratory distress and asthma are recruited from the emergency department or clinics at Children's Hospital Oakland. Study drug is administered as a one-time dose in the emergency department. Arginine or placebo is continued every 8 hours for patients admitted to the hospital. Primary outcome measures are admission vs. discharge patient parameters, and length of hospital stay, improvement in clinical asthma scores and oxygen saturations/need for supplemental oxygen use. Plasma amino acids, arginine-to-ornithine ratio, arginase activity, nitric oxide metabolites (in serum, exhaled breath and urine), PLA2, cytokines, inflammatory biomarkers, genetic modifiers and peak flows are followed.

EXAMPLE 6: ARGININE MONOTHERAPY

[00129] Although Chambers et al. "Effect of nebulised L- and D-arginine on exhaled nitric oxide in steroid naive asthma." Thorax. 2001 Aug;56(8):602-6. reported that administration of inhaled L-Arg to asthma patients induced bronchoconstriction, with exhaled NO decreasing with acute bronchoconstriction, and returning to baseline with the resolution of bronchoconstriction, similar bronchoconstriction occurred with their control test using an alternate amino acid. It is likely that the acute bronchoreaction was due to irritation of the inhalant itself, rather than arginine. Irritation can be avoided by careful selection of a non-irritating inhalant and/or selection of formulation components that do not cause significant irritation upon inhalation (i.e., a low irritant or non-irritating formulation). Such issues can also be avoided by administration of arginine by a route other than inhalation, e.g., by oral or intravenous administration.

[00130] The effects of arginine supplementation on pulmonary function tests is evaluated by administering supplemental arginine (oral or intravenous) alone or in conjunction with magnesium and/or an arginase inhibitor to patients with a known diagnosis of asthma, defined as ≥ 3 wheezing episodes and a past history of asthma medication usage (e.g., bronchodilators, steroids, inhaled steroids, or leukotriene inhibitors etc). Pulmonary function tests are performed before and after a single dose of arginine (0.1 gram/kg to a max of 10 grams).

[00131] One patient has already been enrolled in this study. A single dose of oral arginine (0.1gm/kg) was administered. Pulmonary function tests were determined prior to treatment and 2 hours after arginine supplementation. Although supplemental arginine did not significantly effect spirometry (except FIF 50% - inducing a 23% improvement), and had minimal effect on lung volumes, treatment had an impressive impact on airway resistance within 2 hours (*Raw* decreased by 22% and *Gaw* increased by 28%). Since increased airway resistance is a significant problem during an acute exacerbation of asthma, a benign therapy that decreases airway resistance, likely through smooth muscle relaxation, benefits patients with asthma. Also of interest, the patient's oxygen saturation by venous blood gas increase from 85 to 92%.

[00132] Even greater benefits can appreciated after more than 2 hours post treatment or when used in combination with standard of care asthma therapy such as bronchodilators and steroids.

REFERENCES CITED

1. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nat Med 1987; 327:524-526.

2. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993; 329:2002-2012.

3. Kam PC, Govender G. Nitric oxide: Basic science and clinincal applications. Anaesthesia 1994; 49:515-521.

4. Zoritch B. Nitric oxide and asthma. Arch Dis Child 1995; 72:259-262.

5. Gaston B, Drazen JM, Loscalzo J, Stamler J. The biology of nitrogen oxides in the airways. Am J Respir Crit Care Med 1994; 149:538-551.

6. Nathan C, Shiloh M. Reactive oxygen and nitrogen intermediates in the relationship between
mammalian hosts and microbial pathogens. Proc Natl Acad Sci 2000; 97:8841-8848.

7. Xia Y, Dawson V, Dawson T, Snyder S, Zweier J. Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. Proc Natl Acad Sci 1996; 93:6770-6774.

8. Dias-Da-Motta P, V. A, Muscara M, Saad S. The release of nitric oxide and superoxide anion by neutrophils and mononuclear cells from patients with sickle cell anaemia. Brit J Haematol 1996; 93:333-340.

9. Demiryurek A, Dakici I, Danzik I. Peroxynitrite: A putative cytotoxin. Pharm Toxicology 1998; 82:113-117.

10. Bowton D, Seeds M, Fasano M, Goldsmith B, Bass D. Phospholipase A2 and arachidonate increase in bronchoalveolar lavage fluid after inhaled antigen challenge in asthmatics. Am J Respir Crit Care Med 1997; 155:421-425.

11. Holgate S. Asthma genetics: waiting to exhale. Nat Genet 1997; 15:227-229.

12. Hamid Q, Springall DR, Riveros-Morena V, Chanez P, Howarth P, Redington A, Bousquet J, Godard P, Holgate S, Polak JM. Induction of nitric oxide synthase in asthma. Lancet 1993; 342:1510-1513.

13. Nijkamp FP, Folkerts G. Nitric oxide and bronchial hyperresponsiveness. Arch Int Pharmoocodyn 1995; 329:81-96.

14. Kharitonov SA, Yates D, Barnes PJ. Increased nitric oxide in exhaled air of normal human subjects with upper respiratory tract infections. Eur Respir J 1995; 8:295-7.

15. Ricciardolo F, Geppetti P, Mistretta A, Nadel J, Sapienza M, Bellofiore S, Di Maria G. Randomized double-blind placebo-controlled study of the effect of inhibition of nitric oxide synthesis in bradykinin-induced asthma. Lancet 1996; 348:374-377.

16. Meurs H, Schuurman F, Duyvendak M, Zaagsma J. Deficiency of nitric oxide in polycation-induced airway hyperreactivity. Br J Pharmacol 1999; 126:559-562.

17. Sanders S. Nitric oxide in asthma. Am J Respir Cell Mol Biol 1999; 21:147-149.

18. Pieper GM. Review of Alterations in Endothelial Nitric Oxide Production in Diabetes. Protective Role of Arginine on Endothelial Dysfunction. Hypertension 1998; 31:1047-1060.

19. Lerman A, Burnett JC, Higano ST, McKinley LJ, Holmes DR. Long-term L-arginine supplementation improves small-vessel coronary endothelial function in human. Circulation 1998; 97:2123-2128.

20. Perrine SP, Ginder GD, Faller DV, Dover GH, Ikuta T, Witkowska HE, Cai SP, Vichinsky EP, Olivieri NF. A short-term trial of butyrate to stimulate fetal-globin-gene expression in the beta-globin disorders. N Engl J Med 1993; 328:81-6.

21. Maxwell AJ, Cooke JP. 1998. Cardiovascular effects of L-arginine. *In* P. Vallance and C. Baylis, editors. Current Opinion in Nephrology and Hypertension. 133.

22. Creager MA, Gallagher SJ, Girerd XJ, Coleman SM, Dzau VJ, Cooke JP. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. J Clin Invest 1992; 90:1248-53.

23. Drexler H, Zeiher AM, Meinzer K, Just H. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. Lancet 1991; 338:1546-50.

24. Folkerts G, Van der Linde HJ, Nijkamp FP. Virus-induced airway hyperresponsiveness in guinea pigs is related to a deficiency in nitric oxide. J Clin Invest 1995; 94:26-30.

25. Solomons C, Cotton CK, Dubois R. The use of buffered L-arginine in the treatment of cystic fibrosis. Pediatr 1971; 47:384-390.

26. Solomons C, Hathaway W, Cotton E. L-arginine, the sickling phenomenon, and cystic fibrosis. Pediatr 1972; 49:933.

27. Knight J, Murphy TM, Browning I. The lung in sickle cell disease. Pediatr Pulmonol 1999; 28:205-216.

28. Gladwin M, Schechter A. Nitric oxide therapy in sickle cell disease. Semin Hematol 2001; 38:333-342.

29. Lopez da Mata P, Neuparth N, Carmo M, Caires I, Macedo P, Rendas A. 1998. How does nitrates in blood correlated to exhaled levels in asthma? . European Respiratory Conference, Geneva, Switzerland.

30. Rees DC, Cervi P, Grimwade D, O'Driscoll A, Hamilton M, Parker NE, Porter JB. The metabolites of nitric oxide in sickle-cell disease. Br J Haematol 1995; 91:834-7.

31. Morris CR, Kuypers FA, Larkin S, Vichinsky E, Styles L. Patterns of arginine and nitric oxide in sickle cell disease patients with vaso-occlusive crisis and acute chest syndrome. J Pediatr Hematol Oncol 2000; 22:515-520.

32. Minter K, Gladwin M. Pulmonary complications of sickle cell anemia. A need for increased recognition, treatment, and research. Am J Respir Crit Care Med 2001; 164:2016-2019.

33. Morris CR, Kuypers FA, Larkin S, Sweeter N, Simon J, Vichinsky EP, Styles L. Arginine therapy: A novel strategy to increase nitric oxide production in sickle cell disease. Brit J Haematol 2000; 111:498-500.

34. Morris C, Morris S, Jr., Hagar W, van Warmerdam J, Claster S, Kepka-Lenhart K, Machado L, Kuypers F, Vichinsky E. Arginine Therapy: A new treatment for pulmonary hypertension in sickle cell disease? Am J Respir Crit Care Med 2003; 168:63-69.

35. Boucher JL, Moali C, Tenu JP. Nitric oxide biosynthesis, nitric oxide synthease inhibitors, and arginase competition for L-arginine utilization. Cell Mol Life Sci 1999; 55:1015-1028.

36. Morris SM, Jr. , Kepka-Lenhart D, Chen L. Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. Am J Physiol Endocrinol Metab 1998; 275:740-747.

37. Mori M, Gotoh T. 2000. Relationship between arginase activity and nitric oxide production. *In* L. Ignarro, editor. *Nitric Oxide. Biology and Pathology*. Academic Press, San Diego. 199-208.

38. Waugh W, Daeschner C, Files B, Gordon D. Evidence that L-arginine is a key amino acid in sickle cell anemia - a preliminary report. Nutritional Research 1999; 19:501-518.

39. Meurs J, McKay S, Maarsingh H, Hamer M, Macic L, Molendijk N, Zaagsma J. Increased arginase activity underlies allergen-induced deficiency of cNOS-dervied nitric oxide and airway hyperresponsiveness. Br J Pharmacol 2002; 136:391-398.

40. Morris CR, Kuypers A, Vichinsky E, Kepka-Lenhart D, Morris SM, Jr. 2002. Elevated serum arginase activity in patients with sickle cell disease and pulmonary hypertension. . The 30th Anniversary of the National Sickle Cell Program, Washington, DC.

41. Morris SM, Jr. Regulation of enzymes of the urea cycle and arginine metabolism. Annu Rev Nutr 2002; 22:87-105.

42. Morris SM, Jr. 2000. Regulation of arginine availability and its impact on NO synthesis. . Nitric Oxide. Biology and Pathobiology. Academic Press, San Diego. 187-197.

43. Mori M, Gotoh T. Regulation of nitric oxide production by arginine metabolic enzymes. Biochem Biophys Res Commun 2000; 275:715-719.

44. Kershenobich D, Fierro F, Rojkind M. The relationship between the free pool of proline and collagen content in human liver cirrhosis. J Clin Invest 1970; 49:2246-2249.

45. Albina J, Abate J, Mastrofrancesco B. Role of ornithine as a proline precursor in healing wounds. J Surg Res 1993; 55:97-102.

46. Endo M, Oyadomari S, Terasaki Y, Takeya M, Suga M, Mori M, Gotoh T. Induction of arginase I and II in bleomycin-induced fibrosis of mouse lung. Am J Physiol Lung Cell Mol Physiol 2003; 285:L313-L321.

47. Tanaka H, Masuda T, Tokuoka S, Komai M, Nagao K, Takahashi Y, Nagai H. The effect of allergen-induced airway inflammation on airway remodeling in a murine model of allergic asthma. Inflamm Res 2001; 50:616-624.

48. Elias J, Zhu Z, Chupp G, Homer R. Airway remodeling in asthma. J Clin Invest 1999; 104:1001-1006.

49. Elias J, Lee C, Zheng T, Ma B, Homer R, Zhu Z. New insights into the pathogenesis of asthma. J Clin Invest 2003; 111:291-297.

50. Busse W, Lemanske R. Asthma. N Engl J Med 2001; 344:350-362.

51. Kurosawa M, Shimizu Y, Tsukagoshi H, Ueki M. Elevated levels of peripheral-blood, naturally occurring aliphatic polyamines in bronchial asthmatic patients with active symptoms. Allergy 1992; 47:638-643.

52. Sward K, Pato M, Nilsson B, Nordstrom I, Hellstrand P. Polyaminines inhibit myosin phosphatase and increase LC20 phosphorylation and force in smooth muscle. Am J Physiol 1995; 269:C563-C571.

53. Nilsson B, Hellstrand P. Effects of polyamines on intracellular calcium and mechanical activity in smooth muscle of guinea-pig taenia coli. Acta Physiol Scand 1993; 148:37-43.

54. Hoet P, Nemery B. Polyamines in the lung: polyamine uptake and polyamine-linked pathological or toxicological conditions. Am J Physiol Lung Cell Mol Physiol 2000; 278:L417-L433.

55. Stuehr DJ, Kwon N, Nathan CF, Griffith OW, Felman PL, Wiseman J. N-Hydroxyl-L-arginine is an intermediate in the biosynthesis of nitric oxide for L-arginine. J Biol Chem 1991; 266:6259-6263.

56. Kumar A, Brar R, Wang P, Dee L, Skorupo G, Khadour F, Schulz R, Parrillo J. Role of nitric oxide and cGMP in human septic serum-induced depression of cardiac myocyte contractility. Am J Physiol 1999; 276:265.

57. Zeballos A, Bernstein R, Thompson C, Forfia P, Seyedi N, Kaminiski R, Wolin M, Hintze T. Pharmocodynamics of plasma nitrate/nitrite as an indication of nitric oxide formation in conscious dogs. Circulation 1995; 91:2982.

58. Miller V, Lewis D, Rud K, Offord K, Croghan I, Hurt R. Plasma nitric oxide before and after smoking cessation with nicotine nasal spray. J Clin Pharmacol 1998; 38:22.

59. Nelson BV, Sears S, Woods J. Expired nitric oxide as a marker for childhood asthma. J Pediatr 1997; 130:423-427.

60. Baraldi E, Azzolin NM, Zanconato S, Dario C, Zacchello F. Corticosteriods decrease exhaled nitric oxide in children with acute asthma. J Pediatr 1997; 131:381-385.

61. Styles LA, Schalkwijk CG, Aarsman AJ, Vichinsky EP, Lubin BH, Kuypers FA. Phospholipase A2 levels in acute chest syndrome of sickle cell disease. Blood 1996; 87:2573-8.

62. Li J. Mechanisms of asthma. Current Opinions in Pulmon Med 1997; 3:10-16.

[00133] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and

scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

What is claimed is:

1. A method of treating a subject having elevated arginase as a symptom or cause of a disorder, the method comprising:

administering to a subject in need of therapy an amount of L-arginine and an amount of an arginase inhibitor, said administering being effective to enhance arginine bioavailability in the subject thereby treating the subject.

2. The method of claim 1, wherein the subject is an asthmatic.

3. The method of claim 1, wherein the subject has sickle cell disease.

4. The method of claim 1, wherein the subject has pulmonary hypertension.

5. The method of claim 1, wherein the arginase inhibitor is N(omega)-hydroxy-nor-L-arginine (NOHA), N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) , 2(S)-amino-6-boronohexanoic acid (ABH), S-(+)-Amino-6-iodoacetamidohexanoic acid, S-(+)-Amino-5-iodoacetamidopentanoic acid, L-norvaline, or L-HOArg.

6. The method of claim 1, wherein the arginase inhibitor is NOHA.

7. The method of claim 1, wherein said administering further comprises administering an amount of nitric oxide (NO).

8. A method of treating asthma symptoms in a subject, the method comprising:

administering L-arginine to a subject having or at risk of asthma, said administering being effective to enhance arginine bioavailability in the subject thereby treating asthma symptoms in the subject.

9. The method of claim 8, wherein the method further comprises administration of magnesium.

10. The method of claim 8, wherein the method further comprises administration of an arginase inhibitor.

11. A method of treating pulmonary hypertension in a subject, the method comprising:

administering L-arginine and an arginase inhibitor to a subject having or at risk of pulmonary hypertension, said administering being effective to enhance arginine bioavailability in the subject thereby treating asthma symptoms in the subject.

12. The method of claim 11, wherein the subject has sickle cell disease.

13. The method of claims 11 or 12, wherein the arginase inhibitor is N(omega)-hydroxy-nor-L-arginine (NOHA), N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) , 2(S)-amino-6-boronohexanoic acid (ABH), S-(+)-Amino-6-iodoacetamidohexanoic acid, S-(+)-Amino-5-iodoacetamidopentanoic acid, L-norvaline, or L-HOArg.

14. The method of claims 11 or 12, wherein the arginase inhibitor is NOHA.

15. The method of claims 11 or 12, wherein said administering further comprises administering an amount of nitric oxide (NO).

16. A method of treating a subject having decreased nitric oxide bioavailability as a symptom or cause of a disorder, the method comprising:

administering to a subject in need of therapy an amount of L-arginine and an amount of magnesium, said administering being effective to enhance arginine bioavailability in the subject thereby treating the subject.

17. The method of claim 16, wherein the subject is an asthmatic.

18. The method of claim 16, wherein the subject has sickle cell disease.

19. The method of claim 16, wherein the subject has pulmonary hypertension.

20. The method of claim 16, wherein the decreased nitric oxide bioavailability is a result of decreased arginine in the subject.

21. A composition comprising L-arginine, an arginase inhibitor, and a pharmaceutically acceptable excipient.

22. A composition comprising L-arginine and a pharmaceutically acceptable magnesium salt, and a pharmaceutically acceptable excipient.

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(71) Applicant (*for all designated States except US*): CHILDREN'S HOSPITAL & RESEARCH CENTER AT OAKLAND [US/US]; 747 52nd Street, Oakland, CA 94609 (US).

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): MORRIS, Claudia R. [US/US]; 1595 Huston Road, Lafayette, CA 94549 (US).

(74) Agent: FRANCIS, Carol, L.; Bozicevic, Field & Francis LLP, 1900 University Avenue, Suite 200, East Palo Alto, CA 94303 (US).

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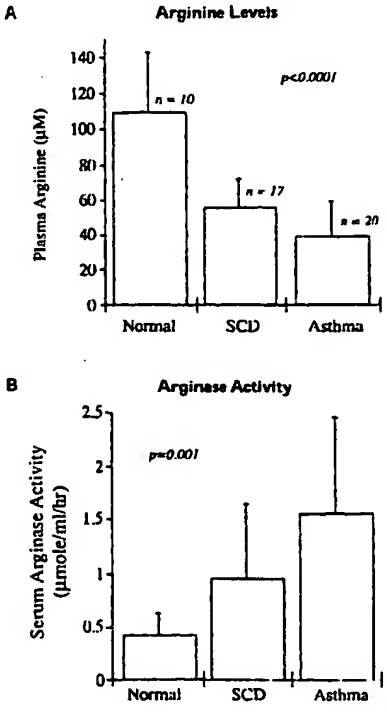
[Continued on next page]

(54) Title: TREATMENT OF CONDITIONS ASSOCIATED WITH DECREASED NITRIC OXIDE BIOAVAILABILITY, INCLUDING ELEVATED ARGINASE CONDITIONS

(57) Abstract: The invention features methods and compositions for treatment of conditions associated with decreased nitric oxide bioavailability, such as a condition associated with elevated arginase activity, using an arginine-based therapy, including combination therapy with an arginase inhibitor and/or magnesium.



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TREATMENT OF CONDITIONS ASSOCIATED WITH DECREASED NITRIC OXIDE BIOAVAILABILITY, INCLUDING ELEVATED ARGINASE CONDITIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the priority benefit of U.S. provisional application serial no. 60/447,373, filed February 14, 2003, which application is incorporated by reference herein in its entirety.

GOVERNMENT RIGHTS

[0002] This invention was made with government support under federal grant nos. RR01271-19 and HL-04386-01 awarded by the National Institutes of Health. The United States Government may have certain rights in this invention.

FIELD OF THE INVENTION

[0003] The present invention is in the field of therapy for conditions associated with elevated arginase as described herein, including asthma, sickle cell disease, and pulmonary hypertension.

BACKGROUND OF THE INVENTION

[0004] L-Arginine (Arg) is a conditionally essential amino acid, naturally found in dietary protein. It is converted to nitric oxide (NO) (1, 2) a potent vasodilator (1-3) and bronchodilator (4, 5), by a family of enzymes known as nitric oxide synthase (NOS). NO is an essential molecule that plays a role in a broad range of functions from vascular regulation, neurotransmission (2), host defense, and cytotoxicity (6) to physiologic control of airways (5). Under conditions of low L-arginine concentration, nitric oxide synthase is uncoupled and reduces oxygen (O_2) to superoxide (O_2^-) instead of generating nitric oxide (7,8). Nitric oxide reacts rapidly with superoxide to form reactive nitric oxide species (*RNOS*) that could lead to worsening inflammation, oxidative stress and cellular damage (9).

[0005] Complex interactions among cellular components of the immune system, endocrine factors, growth factors and cytokines contribute to pathophysiology of asthma. The contribution of some cytokines (IL-1α, IL-1β, IL-3, IL-4, IL-6, IL-11, TNF-α, γγ- interferon, IL-6, M-CSF) have been well studied in asthma. Secretory phospholipase A2 (sPLA2), which is involved in the pathway of leukotriene synthesis is elevated in broncheoalveolar lavage of

antigen-challenged asthmatics (10). The genetic predisposition of asthma is now well recognized (11).

[0006] Recently, expression of inducible NO synthase, the enzyme that catalyzes the production of NO from L-Arg, has been found in the epithelium of asthmatic patients but not in healthy non-asthmatic patients. (12, 13). Asthmatics have exhaled air NO levels that are 3.5 times higher than non-asthmatics, which are correlated with decrease in FEV_1 and are affected by therapy (14). Blocking of NO production by L-Arg analogues results in an increase in allergen-induced bronchoconstriction (15). A deficiency of NO is involved in airway hyperreactivity (16). Although asthma is clearly a multifactorial disease, there is some evidence that NO may play an important role in disease pathogenesis (17). For reviews, see, e.g., Dweik Cleve Clin J Med. 2001 Jun;68(6):486, 488, 490, 493; Gianetti et al. Eur J Clin Invest. 2002 Aug;32(8):628-35.

[0007] Arginase is an enzyme that hydrolyzes Arg to produce ornithine and urea, (35) however, in the presence of nitric oxide synthase (NOS), arginine is converted to nitric oxide (NO) and citrulline (2). The expression of arginase can be induced by a variety of cytokines involved in the inflammatory process (26), particularly the Th2 cytokines. (37). Increased serum arginase activities have been reported in patients with SCD at steady-state (38), as well as in an asthma animal model (39). Arginase activity is elevated in SCD patients with pulmonary disease (34, 40). Plasma arginase activity appears to be related to hemolysis, associated with several markers of hemolytic severity, including LDH ($r=0.44$, $p<0.001$), AST ($r=0.39$, $p<0.002$), reticulocyte count ($r=0.25$, $p<0.001$), and Hct ($r= -0.25$, $p<0.001$) (Morris et al, Erythrocyte arginase release during hemolysis contributes to endothelial dysfunction and pulmonary hypertension, 27th Annual Meeting of the National Sickle Cell Disease Program, Los Angeles, CA; April 2004).

[0008] Arginase controls the metabolism of arginine into ornithine, which in turn gives rise to proline and polyamines (37, 41-43). These downstream products of arginase activity may play a significant role in the pathogenesis of asthma, pulmonary hypertension and other inflammatory conditions, since proline is involved in collagen formation (44, 45) and lung fibrosis (46), processes that occur in airway wall thickening and airway remodeling (47-50).

[0009] Elevated levels of polyamines have been reported in the serum of asthmatic patients (51). Polyamines have contractile activity on smooth muscle (52, 53), and are present in multiple cell types in the lung, including airway epithelium, smooth muscle cells and macrophages (54). Since proline and hydroxyproline (47) are amino acids involved in collagen deposition, and polyamines affect multiple processes, including cell survival, cell proliferation and mucus production (52, 53), they may play a role in lung pathology.

[0010] Arginine, a safe dietary supplement, has already demonstrated potential for therapeutic utility in several disease processes.(18-23). In animal studies, inhalation of low doses of L-Arg has completely blocked hyperresponsiveness of reactive airways (13, 24), and inhaled L-Arg also improves pulmonary functions of cystic fibrosis patients (CF) (25,26). When tested in a mouse model of allergic asthma, oral administration of L-Arg was reported to aggravate allergen-induced eosinophilic airway inflammation (Takano et al. J Pharmacol Exp Ther 1998 Aug;286(2):767-71).

[0011] Use of L-Arg is suggested for treatment of cystic fibrosis (Busch-Petersen et al. Z Erkr Atmungsorgane 143:140-7 (1975)); treatment of exercise induced pulmonary hemorrhage in horses (US Pat. No. 6,027,713); and treatment of pulmonary hypertension (U.S. Pat. Nos. 5,217,997; 6,127,421; Nagaya et al. Am J Respir Crit Care Med 163:887-81 (2001); Cheng et al. Hua Xi Yi Ke Da Xue Xue Bao 27:68-70 (1996)).

[0012] Use of NO to treat asthma is discussed in Nakagawa et al. J Pediatr. 2000 Jul;137(1):119-22;and Rossaint et al. Eur Heart J 1993 Nov;14 Suppl I:133-40). The arginase inhibitor N-hydroxy-L-arginine (NOHA) has been tested in a model of asthma (see, e.g., Meurs et al., Br J Pharmacol Jun 2002, 136(3):391-8, describing administration of an arginase inhibitor in a guinea pig model of allergic asthma; and Meurs et al. Br J Pharmacol 130:1793-8 (2000, describing arginase inhibitors in a perfused guinea pig trachea model)). Use of NO to treat pneumonia has been discussed (see, e.g., Kimura et al. Pediatr Int 2002 Aug;44(4):451-2; Ho et al. J R Soc Med 2002 Jan;95(1):35-7; Bugge et al. Eur J Anaesthesiol 2000 Apr;17(4):269-72; Hoehn et al. Respiration 1998;65(6):477-80; Blomqvist et al. Acta Anaesthesiol Scand 1993 Jan;37(1):110-4; Jean et al. Crit Care Med 2002 Feb;30(2):442-7 and Kannan et al. Indian J Pediatr 1998 May-Jun;65(3):333-45).

[0013] Although early investigators warned of the deleterious impact of nitric oxide in sickle cell disease (SCD) (27), more recent studies support its protective function (28). Similar to asthmatic patients (29), SCD patients also have elevated NO_x levels at baseline (30). Serum L-Arg and NO_x levels fall, however, during the vaso-occlusive complications of SCD, (31) with lowest levels found during acute chest syndrome (pneumonia). Most SCD patients with pulmonary disease have a component of reactive airways that respond to bronchodilators, even though they often do not demonstrate the classical wheezing on physical exam that is usually associated with asthma. Asthma in SCD is often unrecognized and undertreated, and occurs in 30-60% of patients (32). Clinical trials of arginine therapy are now underway for SCD (33, 34).

[0014] Magnesium, which can be a dietary supplement, has been described as an adjuvant in combination therapy of asthma with salbumatol (Hughes et al, Lancet 2003;361:2114-7) or as

an asthma intravenous monotherapy (Gurkan et al, Eur J Emerg Med 1999;6:201-5). Magnesium has also been suggested in infusion therapy of neonatal pulmonary hypertension (Patole S & Finer N, Magnes Res 1995;8:373-88). The effects of oral magnesium in an animal model of pre-eclampsia has been reported (Pandhi et al, Indian J Exp Biol 2002;40:349-51) and other disease processes that involve endothelial dysfunction (Volpe et al, Scand Cardiovasc J 2003; 37:288-96). Magnesium-induced vasodilation has been reported in animal models of other conditions that involve endothelial-derived nitric oxide (Teragawa et al, Magnes Res 2002;15:241-6, describing the effects of magnesium in an in vitro canine coronary artery model of endothelial dysfunction). Combined therapy of magnesium and inhaled nitric oxide has shown some promise in an animal model of pulmonary hypertension (Haas et al, Pediatr Int 2002;44:670-4).

[0015] Despite the advances in the field with respect to therapies for conditions such as asthma and sickle cell disease, new therapies are of considerable interest and importance. Furthermore, therapies based upon a more insightful understanding of the underlying mechanisms of these diseases is needed so as to provide a more rationale approach to therapy.

[0016] There is a need in the field for improved or alternative therapies for treatment of conditions such as asthma. The present invention addresses these needs.

Literature

[0017] U.S. Pat. Nos. 5,217,997; 6,387,890; 4,507,314; 6,359,007; 6,646,006; 6,165,975.

[0018] American Society of Hematology Meeting, San Diego Dec 2003; Morris et al, Blood 2003;102:763a (abstr2818); Inselman et al. "Alterations in plasma amino acid levels in children with asthma: a preliminary investigation." Pediatr Pulmonol. 1986 May-Jun;2(3):163-9; Jorens et al. "L-arginine-dependent nitric oxide synthase: a new metabolic pathway in the lung and airways." Eur Respir J. 1993 Feb;6(2):258-66; Vercelli "Arginase: marker, effector, or candidate gene for asthma" J Clin Invest. 2003 Jun;111(12):1815-7 and Zimmermann et al. "Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis." J Clin Invest. 2003 Jun;111(12):1863-74 relate to microarray analysis of the expression profiles of lung tissue in two murine models of asthma revealed high levels of arginase I and arginase II activity, in association with IL-4 and IL-13 overexpression. Haas et al, "Nitric oxide further attenuates pulmonary hypertension in magnesium-treated piglets" Pediatr Int 2002;44:670-4.

[0019] Meurs et al. "Arginase and asthma: novel insights into nitric oxide homeostasis and airway hyperresponsiveness." Trends Pharmacol Sci. 2003 Sep;24(9):450-5 provides a review

in which the authors proposed that a relative deficiency of NO caused by increased arginase activity and altered L-arginine homeostasis is a major factor in the pathology of asthma.

[0020] Sapienza et al. "Effect of inhaled L-arginine on exhaled nitric oxide in normal and asthmatic subjects." Thorax. 1998 Mar;53(3):172-5 reports that inhaled L-Arg increased exhaled NO in a dose-dependent fashion, with the cumulative effect of L-arginine on NO in asthmatic subjects being significantly higher than in non-asthmatics. This report concluded that L-Arg may have therapeutic potential in diseases in which there is defective production of NO, but in asthma it may amplify the inflammatory response in the airways.

[0021] De Gouw et al. "Effect of oral L-arginine on airway hyperresponsiveness to histamine in asthma." Thorax. 1999 Nov;54(11):1033-5 concludes that oral L-arginine does not influence airway hyperresponsiveness to histamine as reflected by PC(20), although the dose-response slope is slightly reduced in patients with asthma, thus indicating only marginal, clinically unimportant limitation of NO synthase substrate in asthma.

[0022] Chambers et al. "Effect of nebulised L- and D-arginine on exhaled nitric oxide in steroid naive asthma." Thorax. 2001 Aug;56(8):602-6. reported that administration of inhaled L-Arg to asthma patients induced bronchoconstriction, with Exhaled NO decreasing with acute bronchoconstriction, and returning to baseline with the resolution of bronchoconstriction. Exhaled NO increased following the administration of both L-arginine and D-arginine.

SUMMARY OF THE INVENTION

[0023] The invention features methods and compositions for treatment of conditions associated with decreased nitric oxide bioavailability, such as a condition associated with elevated arginase activity, using an arginine-based therapy, including combination therapy with an arginase inhibitor and/or magnesium.

[0024] The invention is advantageous in that, where the invention contemplates administration of arginine in combination with an arginase inhibitor, the invention can avoid the need to administer higher doses of arginine that may otherwise be needed to treat conditions associated with elevated arginase activity. In short, where elevated arginase increases utilization of arginine, higher doses of arginine would be required to overcome this phenomenon in an arginine monotherapy. Administration of an arginase inhibitor in conjunction with arginine can lower therapeutic dose requirements of arginine. A large dose of arginine, e.g., up to 10 pills, three times a day, that may otherwise be required without combination therapy with an arginase inhibitor is a very large hindrance to achieving therapeutic goals, largely due to poor patient compliance.

[0025] Administration of arginine to a patient having elevated arginase levels leads to increased production of ornithine. Plasma ornithine levels strongly correlated to proline levels in asthmatic patients ($r = 0.75$, $p<0.0001$, $n = 26$) . The administration of an arginase inhibitor together with arginine will have the added benefit of decreasing the downstream by-products of ornithine metabolism, e.g., proline and polyamines, both of which are associated with pulmonary and cardiovascular pathology through airway remodeling, lung fibrosis and vascular smooth muscle proliferation. This invention will provide substrate for nitric oxide production, while limiting production of metabolites of arginase activity that would otherwise likely contribute to disease pathology.

[0026] Ornithine also decreases arginine bioavailability through competitive inhibition since arginine and ornithine use the same transporter molecules. In short, elevated arginase activity decreases arginine bioavailability. Arginine administered with an arginase inhibitor maximizes arginine bioavailability even in the context of elevated arginase levels.

[0027] Still another advantage of the invention is that, compared to administration of arginase inhibitor alone, is that arginase inhibitors are quite expensive. Administration of arginine, which is relatively inexpensive, in conjunction with an arginase inhibitor allows for administration of relatively reduced amounts of expensive arginase inhibitors. In short, administration of arginine and arginase inhibitors will be more effective, and a less expensive therapy.

[0028] Another advantage is that the invention avoids the problem that arginine bioavailability remains limited by its low concentration, even in the presence of an arginase inhibitor. Low arginine concentration leads to the uncoupling of nitric oxide synthase (NOS) and superoxide production in lieu of nitric oxide. The Km for arginine transport on the cationic amino acid molecules is around 100 μM; thus reversing the arginine deficiency while maximizing arginine bioavailability and limiting alternate routes of metabolism as per the present invention provide for an improved means for achieving therapeutic goals.

[0029] These and other advantages will be apparent to the ordinarily skilled artisan upon reviewing the present specification.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] Figure 1 is a graph showing plasma Arginine concentration (Panel A) and arginase activity (Panel B) in normal non-asthmatic controls (*Normal*, n=10) vs. SCD patients with PHT (*SCD*, n=17), vs. patients with asthma (*Asthma*, n=20). Arginine levels are low and arginase

activity is elevated in patients with asthma and in SCD patients with pulmonary hypertension compared to normal controls ($p < 0.0001$).

[0031] Figure 2 is a graph showing the change in plasma arginine levels from initial presentation to the emergency department (*Admit*) vs. the day of hospital discharge (*D/C*) in asthmatic children (four patients) requiring hospitalization. Low arginine levels rise significantly as clinical condition improved ($p \leq 0.05$).

[0032] Figure 3 is a graph demonstrating changes in plasma arginine and ornithine concentration (Panel A; closed circles, arginine levels; open circles, ornithinine levels) , arginase activity and nitric oxide metabolites (Panel B; closed circles arginase activity; open circles, serum nitric oxide metabolites (NOx)) during hospitalization in a representation four-year old boy with status asthmaticus.

[0033] Figure 4 is a schematic illustrating competition of arginase with nitric oxide synthase for available L-arginine substrate. Downstream by-products of arginase activity are compounds that likely contribute to disease pathogenesis.

DEFINITIONS

[0034] "Arginine" or "Arg" or "L-Arg" as used herein refers to naturally occurring or synthetically produced L-arginine.

[0035] "Arginase" as used herein refers to an enzyme that mediates conversion of L-Arg into ornithine and urea, and is meant to encompass any or all relevant arginase types, including, for example, arginase type I, arginase type II, and the like.

[0036] "Arginase inhibitor" refers to an agent, such an organic compound or anti-arginase antibody, which agent can be either naturally-occurring or synthetic, which agent affects activity of an arginase (e.g., arginase type I, arginase type II, or both) in catalysis of L-Arg into ornithine and urea. For example, an antibody which binds arginase can affected arginase activity by interfering with arginase binding to its substrate or by promoting clearance of arginase from the subject's circulation. Production of arginase antibodies are well within the skill of the ordinary artisan, and appropriate arginase proteins for production of such antibodies are available.

[0037] As used herein, the terms "treatment," "treating," and the like, refer to obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment," as used herein, covers any treatment of a disease in a mammal,

particularly in a human, and can include: (a) preventing the disease or a symptom of a disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it (e.g., including diseases that may be associated with or caused by a primary disease); (b) inhibiting the disease or condition, i.e., arresting its development; and (c) relieving the disease, i.e., causing regression of the disease.

[0038] The terms "individual," "host," "subject," and "patient" are used interchangeably herein, and generally refer to a mammal, including, but not limited to, primates, including simians and humans., equines (e.g., horses), canines (e.g., dogs), felines, various domesticated livestock (e.g., ungulates, such as swine, pigs, goats, sheep, and the like), as well as domesticated pets and animals maintained in zoos. Treatment of humans is of particular interest.

[0039] Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0040] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0041] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0042] It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for

example, reference to "an arginine inhibitor" includes a plurality of such inhibitor compounds and reference to "the arginase" includes reference to one or more arginase polypeptides and equivalents thereof known to those skilled in the art, and so forth.

[0043] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DETAILED DESCRIPTION OF THE INVENTION

[0044] The present invention is based on the discovery that arginase plays a role in modifying L-Arg bioavailability in SCD, asthma, pulmonary hypertension, and other pathologic conditions of upregulated arginase activity. Increased arginase activity limits arginine bioavailability through its conversion of L-Arg to ornithine and urea, thereby competing with NOS for available L-Arg substrate and regulating nitric oxide (NO) production. Ornithine itself also decreases L-Arg bioavailability, since both L-Arg and ornithine compete for the same transport system for cellular uptake. Downstream by-products of arginase activity, e.g., proline and polyamines have been implicated in lung and cardiovascular pathology, by way of airway remodeling, fibrosis and vascular smooth muscle proliferation. In addition to decreasing NO bioavailability, elevated arginase activity also provides substrate for a pathway which produces metabolites that likely play a role in the pathogenesis of asthma, pulmonary hypertension and other inflammatory conditions.

[0045] There are several possible mechanisms that could lead to increased arginase activity in sickle cell disease. Chronic and acute hemolysis could result in an increased dumping of red blood cell arginase into the circulation. Long-term effects of chronic end organ damage, particularly involving the liver and kidneys, which contain high arginase concentrations, may also lead to leakage of intracellular arginase into the circulation. The inflammatory state of both sickle cell disease and asthma could play a role, as arginase gene expression is upregulated by many cytokines involved in the inflammatory process.

[0046] Without being held to theory, the present invention is based on the hypothesis that arginase plays a role in modifying L-Arg bioavailability in SCD, asthma, pulmonary hypertension, and other pathologic inflammatory conditions that upregulate arginase levels/activity. Increased arginase activity limits arginine bioavailability through its conversion of L-Arg to ornithine and urea, thereby competing with nitric oxide synthase (NOS) for

available L-Arg substrate and interfering with NO production (Figure 4). L-Arg produces nitric oxide (NO) and citrulline (cit) in the presence of the nitric oxide synthase enzyme (NOS) . Nitric oxide release causes vasodilation through the activation of soluble guanylate cyclase (GTP) to the intracellular messenger cyclic GMP (cGMP). Arginase converts L-arginine to ornithine and urea. Both L-arginine and ornithine use the same Cationic Amino Acid Transporter molecule (CAT) for cellular uptake Ornithine can competitively inhibit L-arginine transport into the endothelial cell, thereby limiting substrate availability for nitric oxide synthase and regulating nitric oxide production. N^G-hydroxyl-L-arginine is the intermediate product of the L-arginine-nitric oxide pathway (33, 55), and is a potent inhibitor of arginase activity.

[0047] Accumulation of both intracellular and extracellular N^G-hydroxyl-L-arginine favors the continued conversion of L-arginine to nitric oxide by maintaining adequate arginine availability. The downstream by-products of arginase activity, i.e., proline and polyamines, likely play a role in disease pathogenesis, as they are involved in vascular smooth muscle proliferation as well as airway remodeling (Figure 4). These metabolites may accumulate in serum or plasma as seen in sickle cell patients with pulmonary hypertension. This is a novel model for the pathogenesis of pulmonary hypertension.

[0048] Proline is involved in collagen formation (44, 45) and lung fibrosis (46), processes that occur in airway wall thickening and airway remodeling (47-50). Proline plays an important function in tissue remodeling and normal wound healing (45), however overproduction can lead to pathologic states. Elevated arginase activity can lead to such conditions.

[0049] In an environment of low L-arginine concentration, nitric oxide synthase is uncoupled and reduces oxygen (O_2) to superoxide (O_2^-) instead of generating nitric oxide. Nitric oxide reacts rapidly with superoxide to form reactive nitric oxide species (RNOS) that could lead to oxidative stress and cellular damage. Pathological conditions of increased arginase activity thus would have a negative impact on nitric oxide bioavailability. In short, since both arginase and NOS use Arg as a common substrate, arginase plays a role in regulating nitric oxide (NO) synthesis by modulating L-Arg availability. Decreased arginine bioavailability leads to hyperreactive airways in both SCD and asthma, since it plays a role in bronchodilation. Thus, decreased arginine bioavailability and elevated arginase activity contributes to the disease process. Furthermore, decreased arginine bioavailability leads to pulmonary hypertension in the susceptible patient.

[0050] The data presented herein demonstrate that asthmatic patients exhibit a significant arginine deficiency during acute exacerbations that is even greater than what is observed in

patients with SCD (109.0±33.1 vs. 55.4±16.0 vs. 38.9±20μM in plasma of normal controls vs. SCD patients with pulmonary hypertension vs. asthma, respectively, $p<0.0001$, Figure 1, Panel A). Arginine levels rise significantly by discharge in asthmatics admitted to the hospital (Figure 2). In SCD, this arginine deficiency translates to decreased nitric oxide bioavailability. Arginase activity is elevated in asthmatic patients, (1.6±0.9 vs. 0.95±0.7 vs. 0.427±0.2 μmol/ml/hr, asthma vs. SCD vs. normal controls respectively, $p=0.001$, Figure 1, Panel B).

[0051] In addition, the inflammatory state of the patient's condition can also play a role, as arginase gene expression is upregulated by many cytokines involved in the inflammatory process, particularly the Th2 cytokines. Data presented herein demonstrates elevated sPLA2 levels in serum of asthmatic patients vs. normal controls (4.2±2 vs. 25.9±30, $p<0.05$, normal control vs. asthma) . Besides the basal cytokine production, the additional increase in the serum and local cytokine levels may be induced by activated lymphocytes, monocytes and other inflammatory cells.

[0052] The invention will now be described in more detail.

ARGININE AND ARGINASE INHIBITORS

Arginine

[0053] Arginine as used herein generally refers to L-arginine or "L-Arg". Arginine useful in the invention can be isolated from naturally-occurring sources, provided in an enriched source (e.g., in a foodstuff in which relatively high levels in terms of percent weight is found naturally or is modified to contain such higher levels), or produced by synthetic methods.

[0054] L-Arg can be administered as any physiologically acceptable salt, such as the hydrochloride salt, glutamate salt, nitrite, ascorbate etc . L-Arg can also be administered as a peptide (e.g., poly-L-arginine, or combinations of L-Arg and poly-L-arginine). Oligopeptides of particular interest include oligopeptides of from 2 to 30, usually 2 to 20, preferably 2 to 10 amino acids, having at least 50 mol % of L-arginine, preferably at least about 75 mol % of L-arginine, more preferably having at least about 75 mol % of L-arginine. The oligopeptides can be modified by being ligated to other compounds, which can enhance absorption from the gut, provide for enhancement of NO synthesis or stability, e.g. reducing agents and antioxidants, and the like

Arginase Inhibitors

[0055] A variety of arginase inhibitors can be adapted for use in the present invention. The arginase inhibitor can be a reversible or irreversible arginase inhibitor, or arginase antibody.

Preferably the arginase inhibitor is compatible for use, or can be adapted so as to be compatible for use, in a pharmaceutically acceptable formulation or in a nutraceutical. Exemplary arginase inhibitors include, but are not necessarily limited to, N(omega)-hydroxy-nor-L-arginine (NOHA),N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) , 2(S)-amino-6-boronohexanoic acid (ABH) (see, e.g., US Pat. No. 6,387,890), S-(+)-Amino-6-iodoacetamidohexanoic acid (irreversible); S-(+)-Amino-5-iodoacetamidopentanoic acid (irreversible); L-norvaline, L-HOArg, and the like. NOHA is of particular interest in the present invention.

Magnesium

[0056] Without being held to theory, since magnesium has a role in the L-arginine-nitric oxide pathway and attenuates endothelial dysfunction, combination therapy with arginine (with or without an arginase inhibitor) augments the brochodilatory and vasodilatory properties of magnesium through this pathway. Conditions of associated with decreased nitric oxide bioavailability (e.g., endothelial dysfunction) are amenable to treatment with arginine and magnesium (alone or with an arginase inhibitor). Such combination therapy can have synergistic benefits in treatment of conditions of decreased nitric oxide bioavailability and/or decreased arginine bioavailability.

NO

[0057] NO can be administered in a variety of forms, including, but not limited to inhalation, or as a nitric oxide (NO) donor , and the like. NO gas can be inhaled, while NO donors can be administered in a variety of ways according to the nature of the compound, the manner in which it is formulated, and the like. Exemplary NO donors include, but are not necessarily limited to hydroxyurea is an NO donor, sildenafil, nitrite, however there are many agents that are NO donors.

Formulations

[0058] L-Arg, arginase inhibitors, magnesium, or other agent for administration according to the invention (referred to herein as "the agents" for convenience) can be formulated in a variety of ways suitable for administration according to the methods of the invention. In general, these compounds are provided in the same or separate formulations in combination with a pharmaceutically acceptable excipient(s). A wide variety of pharmaceutically acceptable excipients are known in the art and need not be discussed in detail herein. Pharmaceutically acceptable excipients have been amply described in a variety of publications, including, for example, A. Gennaro (2000) "Remington: The Science and Practice of Pharmacy," 20th edition, Lippincott, Williams, & Wilkins; Pharmaceutical Dosage Forms and Drug Delivery Systems (1999) H.C. Ansel et al., eds., 7th ed., Lippincott, Williams, & Wilkins; and Handbook

of Pharmaceutical Excipients (2000) A.H. Kibbe et al., eds., 3rd ed. Amer. Pharmaceutical Assoc.

[0059] The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are readily available to the public. Moreover, pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are readily available to the public.

[0060] In some embodiments, the agents are formulated separately or in combination, e.g., in an aqueous or non-aqueous formulation, which may further include a buffer (e.g., L-Arg with an arginase inhibitor and/or magnesium, such as L-Arg with an arginase inhibitor, L-Arg with magnesium, L-Arg with both an arginase inhibitor and magnesium, for example). Suitable aqueous buffers include, but are not limited to, acetate, succinate, citrate, and phosphate buffers varying in strength from 5 mM to 100 mM. In some embodiments, the aqueous buffer includes reagents that provide for an isotonic solution. Such reagents include, but are not limited to, sodium chloride, and sugars e.g., mannitol, dextrose, sucrose, and the like. In some embodiments, the aqueous buffer further includes a non-ionic surfactant such as polysorbate 20 or 80.

[0061] Optionally the formulations may further include a preservative. Suitable preservatives include, but are not limited to, a benzyl alcohol, phenol, chlorobutanol, benzalkonium chloride, and the like. In many cases, the formulation is stored at about 4°C. Formulations may also be lyophilized, in which case they generally include cryoprotectants such as sucrose, trehalose, lactose, maltose, mannitol, and the like. Lyophilized formulations can be stored over extended periods of time, even at ambient temperatures.

[0062] In the subject methods, the agents may be administered to the host using any convenient means capable of resulting in the desired therapeutic effect. In general, administration can be by any suitable parenteral (e.g., intravenous, intramuscular, subcutaneous, and the like) or enteral (e.g., oral) route. Thus, the agents can be incorporated into a variety of formulations for therapeutic administration. More particularly, the agents of the present invention can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants and aerosols.

[0063] In pharmaceutical dosage forms, the agents may be administered in the form of their pharmaceutically acceptable salts, or they may also be used alone or in appropriate association,

as well as in combination, with other pharmaceutically active compounds. The following methods and excipients are merely exemplary and are in no way limiting.

[0064] The agents can be formulated into preparations for injection by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

[0065] For oral preparations, the agents can be used alone or in combination with appropriate additives to make tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents. In some embodiments, particularly in the case of L-Arg, the agents can be formulated in the form of a nutriceutical, e.g., as a food product, e.g., admixed with a foodstuff.

[0066] Furthermore, the agents can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. The compounds of the present invention can be administered rectally via a suppository. The suppository can include vehicles such as cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature. Agents can also be provided in sustained release or controlled release formulations, e.g., to provide for release of agent over time and in a desired amount (e.g., in an amount effective to provide for a desired therapeutic or otherwise beneficial effect).

[0067] Unit dosage forms for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit, for example, teaspoonful, tablespoonful, tablet or suppository, contains a predetermined amount of the composition containing one or more inhibitors. Similarly, unit dosage forms for injection or infusion administration may comprise the inhibitor(s) in a composition as a solution in sterile water, normal saline or another pharmaceutically acceptable carrier.

[0068] The term "unit dosage form," as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of the agents calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications

for the unit dosage forms for use in the present invention depend on the particular compound employed and the effect to be achieved, the pharmacodynamics associated with each compound in the host, and the like.

[0069] Dosage forms of particular interest include those suitable to accomplish parenteral (e.g., intravenous, intramuscular, subcutaneous, and the like) or oral administration, as well as dosage forms to provide for delivery by a nasal or pulmonary route (e.g., inhalation), e.g., through use of a metered dose inhaler and the like.

[0070] In general, arginine for use in the invention is formulated in either parenteral or enteral forms, usually enteral formulations, more particularly oral formulations. In one embodiment of particular interest, L-Arg is administered in the form of a dietary supplement, which can be provided as, for example, a drink, powdered drink or foodbar. Where the subject has asthma, administration of the agent (e.g., arginine) in an inhaled formulation that is free of irritants, or by a route other than inhalation (e.g., oral or by injection), may be preferred.

[0071] Arginase inhibitors for use in the invention are formulated for parenteral administration, e.g., by subcutaneous, intradermal, intraperitoneal, intravenous, or intramuscular injection. Administration may also be accomplished by, for example, enteral, oral, buccal, rectal, transdermal, intratracheal, inhalation (see, e.g., U.S. Pat. No. 5,354,934), etc.

[0072] Arginine and arginase inhibitors may be administered as separate dosage forms by the same or different route, or may be formulated as a single dosage form. In one embodiment, arginine and an arginase inhibitor are administered in the form of a capsule, foodbar, or drink, where the two agents may be in separate dosage forms or combined in the same dosage form. In another embodiment, arginine and an arginase inhibitor are provided in the same or different formulation for nebulized delivery. Nebulized delivery may be of particular interest for administration for treatment of asthma and pulmonary hypertension.

[0073] Magnesium is generally be administered as a pharmaceutically acceptable magnesium salt, such as, for example, magnesium sulfate, magnesium chloride or the like. Magnesium can be administered as an oral preparation or medicinal food, an intravenous preparation, and/or it can be nebulized as an inhalant. Exemplary dosing for nebulization includes but is not limited to at least about 3cc (3.2 % soln, 95mg), which can be administered as a one-time dose, a continuous nebulization over one to several hours, or every 5minutes, 10 minutes, 15 minutes, 20 minutes, 30 minutes, hourly, or other dosing schedule as may be medically indicated (e.g., by a clinical practitioner). Exemplary intravenous dosing includes, but is not limited to, at least about 10 mg/kg to about 500 mg/kg, with exemplary and oral dosing of, for example, at least

about 200 gm/day to about 1000 gm/day given as a single dose or divided BID, TID or QID as medically indicated may be used.

Additional agents for combination therapy

[0074] In addition to combination therapy involving administration of L-Arg alone and/or with and an arginase inhibitor, the invention also contemplates administration of additional agents. In one embodiment of particular interest, nitric oxide (NO) donors, and/or NO in the form of inhaled NO gas, is administered to the subject. In the context of treatment of asthma, the therapeutic methods of the invention can further include administration of magnesium and/or anti-inflammatory agents such as, for example, phospholipase inhibitors, particularly cytosolic or secretory phospholipase (PLA, e.g., phospholipaseA2 (PLA2)), leukotriene inhibitors, corticosteroids.

[0075] Additionally, patients with asthma as well as those with sickle cell disease demonstrate deficiencies in many amino acids. Since extracellular arginine deprivation has been shown to influence intracellular amino acid concentrations, improved arginine bioavailability can serve to normalize some of the aberrant amino acid patterns seen in these disease states. However, combination therapy of other deficient amino acids, such as those indicated as deficient in the examples below, in addition to an agent described herein (e.g., arginine and/or an arginase inhibitor and/or magnesium) can also be beneficial and is included in this invention. Exemplary PLA inhibitors that may be useful are described in U.S. Pat. Nos. 6,492,550; 6,443,001; 6,214,876; 5,641,800; and 5,514,704.

[0076] It is well within the skill of the ordinary artisan, given the guidance provided herein, to select a dose and dosage regimen of L-Arg, and/or an arginase inhibitor and/or magnesium to provide for a desired therapeutic or otherwise beneficial effect in the subject. Precise doses and dosage regimens can vary with such factors as, for example, whether L-Arg is administered as a monotherapy or in combination with an arginase inhibitor and/or magnesium and/or other agent, subject-dependent factors (e.g., body metrics (e.g., weight, height, size, body surface area, and the like), health, tolerance to agent and/or formulation, and the like); agent-dependent factors (e.g., pharmacokinetics (e.g., including serum half-life), bioavailability, and the like); dosage regimen-dependent factors (e.g., route of administration, course of therapy, and the like); and dosage form-dependent factors (e.g., formulation, bolus dosage form, sustained release dosage form, and the like). In general, Arg is administered in a dose of are up to 0.1 g/kg body weight BID (twice daily) to TID (three times daily) with a maximum dose of about 30 gms/day. Lower doses can be administered where arginase inhibitor provides for increased

arginine bioavailability, as discussed above. Doses of arginase inhibitor can readily be determined, and in generally are lower amounts than that for arginine.

METHODS OF TREATMENT AND SUBJECTS AMENABLE TO TREATMENT ACCORDING TO THE INVENTION

[0077] Any subject having a condition associated with decreased nitric oxide bioavailability, such as that which results from decreased arginine bioavailability, elevated arginase (e.g., arginase activity and/or arginase levels), or decreased NO bioavailability are amenable to therapy according to the invention. Such therapies include administration of L-Arg (e.g., as a dietary supplement, etc.), which in embodiments of particular interest is administered in conjunction with an arginase inhibitor (e.g., NOHA, arginase antibodies), magnesium, or combinations thereof. For example, magnesium can be administered in conjunction with L-Arg or in addition to a combination therapy of L-Arg and arginase inhibitor. Optionally, NO (e.g., in the form of an inhaled gas or NO donor) can be administered in conjunction with L-Arg monotherapy or combination therapy of L-Arg and arginase inhibitor and/or L-Arg and magnesium. The phrase "in conjunction with" means that an agent is administered prior to, concurrently, or after other substance or therapy.

[0078] The agents (e.g., L-Arg, arginase inhibitor, magnesium, NO) can be administered as separate formulations or, where feasible, as a combined formulation. The agents can be administered at the same time or at different times. Dosages of agents in each of the contexts above can be based upon the various factors as described above. In general, doses may be administered TID (three times a day), BID (twice a day) or QID (four times daily)or QD (daily). For example, the particular regimen for arginase inhibitor (and for arginine) will vary according to a variety of patient factors. For example, where the patient to be treated has sickle cell disease, TID or BID may be of particular interest. For status asthmaticus, therapy may be administered as a one-time dose in the acute setting, or QD, BID, TID, or QID as deemed medially appropriate.

[0079] Exemplary conditions associated with decreased nitric oxide bioavailability and/or elevated arginase levels (relative to non-disease individuals) include, but are not necessarily limited to asthma, sickle cell disease (SCD), pulmonary hypertension (in SCD, neonatal pulmonary hypertension and/or persistent pulmonary hypertension of the newborn, primary hypertension, secondary hypertension), pneumonia, chronic obstructive pulmonary disease (COPD), systemic hypertension, pregnancy related hypertension (pre-eclampsia/eclampsia, arteriosclerosis, diabetes, trauma injury, sepsis, cystic fibrosis, erectile dysfunction, and

hemolytic disorders (where the source of elevated arginase activity is via release from the red blood cell). Conditions amenable to therapy include those that have been previously treated (e.g., as in steroid therapy for asthmatics) or that have not been previously treated ("treatment naïve").

[0080] By "elevated arginase levels" is meant that the subject exhibits a level of arginase activity that is about 20% greater, usually more than about 20% greater, than arginase activity of an average normal subject. Arginase activity measurements in serum or plasma are a special test that is not routinely available. Specialized laboratories can provide this service. Results may vary depending upon the laboratory performing the analysis. Therefore, results must be compared to normal controls (i.e., patients without an inflammatory condition that might be associated with increased arginase activity). Normal, unaffected humans (as reported by Waugh et al, Nutritional Research. 1999. 19;501-518) demonstrate plasma arginase activity levels of 0.2±0.3μM/ml/30 min. The present inventor has observed normal serum arginase activity of 0.4±0.2μM/ml/hr. Thus, arginase activity in plasma and serum of normal controls are low. Levels that are at least about 20% or more above normal are considered elevated. For example, a serum arginase level that is ≥0.6μM/ml/hr would generally be considered an elevated arginase level.

[0081] Asthma is a complex syndrome with many clinical phenotypes that involve a multitude of mechanisms, influenced also by genetic and environmental factors. Individual patient response to asthma therapy also varies, and is likely a reflection of the various mechanisms responsible for disease development and severity. The invention is indicated for those types of asthma that involve elevated arginase activity, decreased arginine bioavailability, and/or limited nitric oxide bioavailability. Included in this group are all varieties of asthma (e.g., allergic asthma, nocturnal asthma, exercise-induced asthma, mild-intermittent, moderate intermittent, moderate persistent, severe persistent, etc). The same is true for the various forms of pulmonary hypertension, and other diseases that manifest with similar clinical symptoms or phenotype but possess underlying mechanistic differences. Altered arginine and nitric oxide bioavailability are likely a common denominator in many of these disease processes, and as such, are amendable to treatment described in this invention.

[0082] This invention may be utilized for acute care during exacerbations of the above described conditions, for treatment of the chronic condition, and/or as prophylaxis to avoid development or progression of the described conditions. Many of these conditions have genetic modifiers that have already been identified that put an individual at risk for developing

certain diseases, and such techniques (including but not limited to HLA testing, microarray analyses, evaluation of genomic polymorphisms etc) may be helpful in identifying patients who would benefit from this invention.

Arginase levels

[0083] Arginase levels and/or arginase activity can be assessed according to methods well known in the art. See, e.g., Morris et al. Am J Physiol Endocrinol Metab 1998;275:740-747. For example, arginase levels can be assessed in blood (e.g., whole blood or serum, plasma, or other blood fraction), bronchoalveolar lavage, or in target organ tissue samples (e.g., found on biopsy). As used herein "detection of arginase" is meant to encompass detection of arginase protein in a sample, detection of activity of arginase in a sample, or both.

[0084] Arginase activity, particularly that present in the serum or plasma of patients, may also be assessed based on the arginine-to-ornithine ratio. This ratio is also helpful in evaluating arginine bioavailability, which is limited by elevated plasma ornithine levels through competitive inhibition of cellular uptake of arginine. The present inventor has found that the arginine/ornithine ratio is significantly lower in sickle cell patient with pulmonary disease (pulmonary hypertension). Likewise, the present inventor has found that the arginine/ornithine ratio is significantly lower in asthmatics, compared to normal controls (0.94 ± 0.5, $n=26$ vs. 1.6 ± 0.6, $n=15$, $p=0.003$).

[0085] In normal control patients studied, arginine levels were usually greater than ornithine levels, such that the ratio often approached 2:1. Such a ratio would avoid a limitation on arginine bioavailability purely on the basis of competitive inhibition, since Arg and ornithine share the same amino acid transporter molecules. Without being held to theory, as the ornithine concentration rises, and the arginine-to-ornithine ratio decreases, arginine bioavailability becomes limited even under conditions of apparently normal arginine concentration. Pathologically elevated arginase activity reduces the arginine-to-ornithine ratio by utilizing arginine (and decreasing that which is available to nitric oxide synthase to make nitric oxide), while hydrolyzing arginine to ornithine, the substrate for proline and polyamine production, metabolites likely involved in disease pathogenesis

[0086] A low arginine-to-ornithine ratio, thus, is a reflection of increased arginase activity. Once this ratio nears or drops below 1, arginine availability for nitric oxide production has reached a competitive disadvantage. An arginine-to-ornithine ratio of less than about 1.2 is considered low. Patients with such a finding, regardless of the disease pathology, may be

treated with the arginine/arginase inhibitor combination therapy, arginine/magnesium combination therapy, or other therapy of the invention.

Assessing therapy

[0087] Following administration of a therapy according to the invention, efficacy can be assess in the patient by, for example, observing an improvement or stabilization in one or more symptoms relevant to the disease being treated. Therapy can also be assessed by assessing arginase levels or activity and/or a normalization of the arginine-to-ornithine ratio. Doses of agents administered can be adjusted in accordance to patient need, e.g., to provide for a decrease of arginase activity levels to within a normal range, e.g., within a range such that arginase levels are not above normal levels more than about 5%, 10%, 15%, or 20%, or a sufficient increase in plasma arginine concentration to the extent that arginine bioavailability is no longer limiting factor for nitric oxide production, ie levels above the Km for arginine transport (>120μM), and a normalization of the arginine-to-ornithine ratio (>1.5).

[0088] Therapy can be assessed by examining improvement in one or more clinical symptoms of disease. Successful therapy is normally considered to be a significant improvement in one or more clinical symptoms after treatment according to the invention as compared to prior to such treatment. In some embodiments, an "effective amount" of L-Arg, or an effective amounts in the context of a combination of L-Arg and an arginase inhibitor, is a dosage that is effective to improve one or more clinical parameters of the condition by at least about 10%, at least about 15%, at least about 25%, at least about 50%, or more, compared to the clinical parameter prior to therapy, or compared with a placebo control or an untreated control. For example, in pulmonary hypertension, clinical parameters assessed can be one or more of: an improvement in mean pulmonary artery systolic pressure as estimated by tricuspid regurgitant jet velocity measured by Doppler-echocardiograpy, improved exercise tolerance as measured by a "6-minute walk"; blood pressure in systemic hypertension, etc),.

[0089] In the context of conditions that affect lung function, the clinical parameters can be, for example, forced inspiratory flow (FIF), forced expiratory flow (FEF), forced vital capacity (FVC), diffusing capacity for carbon monoxide (DLco), and/or the like. For example, in asthma, therapy can be assessed by spirometry, lung volume, airway resistance, and/or oxygen saturation. In patients having pulmonary hypertension, therapy can be assessed using lung function tests, as well as assessing mean pulmonary artery pressure (e.g., at rest and/or with exercise). It should be noted that successful therapy according to the invention includes outcomes where the underlying disease state is not significantly altered, but one or more

clinical symptoms (including symptoms that arise from or are associated with the disease) are treated.

[0090] In the context of sickle cell disease, clinical parameters include, for example one or more of: a decrease in the number of pain crisis, number emergency department visits, number of hospitalizations and/or duration of hospitalization, amount of pain medication use, incidence of and/or occurrence of complications such as skin ulcers, need for transfusion, oxygen use, etc. Also improved pain scores and quality of life assessment tools can be followed.

KITS

[0091] Kits with unit doses of L-Arg formulation, an arginase inhibitor formulation, and/or magnesium formulation (which formulations may be combined or separate as described herein) suitable for use in the methods of the invention are provided. In such kits, in addition to the containers containing the unit doses will be an informational package insert describing the use and attendant benefits of the agents in treating conditions associated with elevated serum arginase activity

[0092] In some embodiments, a subject kit includes a container comprising a formulation comprising a unit dose of L-Arg, an arginase inhibitor, magnesium, or combination thereof, and a pharmaceutically acceptable excipient; and instructions to administer the dosage form according to a desired regimen or exemplary regimen dependent upon the particular condition to be treated, patient age, patient weight, and the like. The instructions can be printed on a label affixed to the container, or can be a package insert that accompanies the container.

[0093] In another embodiment, the agents for administration (e.g., L-Arg, arginase inhibitor, magnesium, NO) are provided in the kit along with materials to facilitate analysis of serum arginase levels in the subject who is a candidate for therapy according to the invention.

EXAMPLES

[0094] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric.

METHODS AND MATERIALS

[0095] The following methods, materials, and patient populations relate to those referred to in the Examples below.

[0096] *Asthma patients.* Patients with asthma presenting to the emergency department and clinics at Children's Hospital and Research Center at Oakland were recruited. Blood samples and exhaled nitric oxide levels (in patients old enough to perform peak flow) are obtained at presentation to the emergency department or clinic, and followed daily during hospitalization for those patients ill enough to require admission.

[0097] Baseline blood was obtained at least 4 weeks after resolution of the acute exacerbation. Blood samples were analyzed for arginine and amino acid levels, arginase activity, and arginine-to-ornithine ratio. Additional analyses that may be performed include analysis of TH-2 cytokines cytokines, VCAM and ICAM, nitric oxide metabolite levels (in blood, breath and urine), genetic markers, IgE, Pla2 levels, RSV (in < 2 year old acutely wheezing) and proteomic analysis. A clinical asthma score routinely used at Children's Hospital Oakland, peak flows (when age appropriate) is obtained, and a symptoms questionnaire (see appendix) is filled out on each patient.

[0098] Well asthmatics (mild intermittent under good control) and non-asthmatic normal controls will also be recruited for comparison. Wheezing infants who do not carry the diagnosis of asthma will also be recruited for participation in this study in order to determine whether elevated arginase, Th2 cytokines and genetic modifiers can differentiate a subgroup of patients likely to develop asthma (as defined by 3 or greater episodes of wheezing). Follow-up phone calls to these families are done in order to determine repeat episodes of wheezing 1 year after enrollment. A paired student *t*-test and ANOVA is used for repeated measurements within the same patient, and an unpaired student *t*-test is used to compare different groups.

[0099] *Sickle cell patients.* Seventeen sickle cell disease patients with documented pulmonary hypertension at steady-state were enrolled in the study. All known patients with pulmonary hypertension receiving care at the Northern California Comprehensive Sickle Cell Center were approached for participation in this analysis. Twelve patients were homozygous for hemoglobin S, three patients had hemoglobin type SC, and two patient had hemoglobin S β-thalassemia. The mean age of patients was 32.7±15 years with a range of 13 to 63 years. There were seven women enrolled. Ten ethnically matched normal non-sickle cell disease volunteers were enrolled as a control group in order to compare amino acid levels and arginase activity. The mean age was 20.6±10 years, ranging from 10 to 34 years. There were four females and

six males enrolled. Pulmonary hypertension was defined as estimated pulmonary artery pressures > 30 mm Hg by echocardiogram (or tricuspid regurgitant jet velocity of greater than 2.5m/sec), > two months duration, not associated with acute chest syndrome. A chart review was performed on all patients to obtain tricuspid regurgitant jet velocity data from previous echocardiograms.

[00100] ***Amino Acid Levels.*** (A complete amino acid panel, including arginine, citrulline, ornithine, and L-arginine analogue asymmetric di-methyl-L-arginine). Quantitative plasma amino acid levels are measured in μmol/L, using a Beckman 6300 amino acid analyzer. The amino acids are separated on an lithium ion exchange column and then reacted with ninhydrin to generate a color response. The data is collected and analyzed using Beckman 32 Karat software, at the Molecular Structure Facility, University of California, Davis, CA.

[00101] ***Arginase***: Arginase-specific activity is determined in plasma by methods previously described. (36)

[00102] ***NOAnalyzer***: Serum is stored at -70° until assayed for nitrate/nitrite/S-NO. NO_x can be measured in serum, plasma or urine according to manufacturer's instructions, using Sievers NOAnalysis software for liquid sampling (Sievers Instruments, Inc., Denver, CO), as previously described.(38-40) Briefly, serum nitrite is measured by acidifying serum to a pH <2.0 to convert nitrite to NO. Serum nitrate is measured by incubating serum with Aspergillus nitrate reductase (Boehringer, Mannheim) to reduce nitrate into nitrite and then convert nitrite into NO by the addition of hydrochloric acid. The NO produced is then injected into the NO analyzer (Sievers, Inc), and the NO content of the sample is determined by measuring the luminescence generated in the presence of ozone. The luminescence measured is directly proportional to the amount of NO injected and, in turn, to the nitrite and nitrate content of the samples. Serum samples can be run immediately, or frozen for later analysis.

[00103] ***Exhaled Nitric Oxide***: Exhaled nitric oxide is measured in exhaled air, using microprocessor-based chemiluminescent NO_x analytical instrumentation, manufactured by Sievers Instruments, Inc. (Denver, CO). The test is easily performed and has been successfully used in many clinical trials. (12, 41, 42) Subjects inhale to total lung capacity from a reservoir bag through a one-way valve (Hans Rudolph, Kansas City, MO) with incoming NO-free air to ensure the absence of environmental NO. Next, the subjects exhale to residual volume into the Teflon tube, which enters into the NO analyzer. The subjects exhale at a pressure of +20 mmHg into the tubing connected to the analyzer. Exhalation at this expiratory pressure without

a nose clip is a maneuver that closes the velum of the posterior nasopharynx and excludes contamination by nasal NO.

[00104] ***Immunofluorescence staining and flow cytometry (FACS) analysis.*** Whole blood samples collected into preservative free heparin is used. Monoclonal antibodies used for staining are: FITC conjugated CD3, CD25, CD69, CD80, CD86, CD95 (Immunotech, Westbrook, ME), PE conjugated CD 154 (CD40L), CD16, CD56, CD63 (Becton Dickinson, San Jose, CA), FITC conjugated CD45RA, CD40 (Coulter, Hialeah, FL), PE conjugated CD45RO (Beckton-Dickinson, CA), PerCP conjugated CD3, CD4, CD8, CD19 (Beckton-Dickinson, San Jose, CA). Two- and three-color analyses are performed on the FACScan (BDIS, Mountain View, CA). 10,000 events are acquired and analyzed.

[00105] ***T cell activation.*** Heparinized blood is diluted 1:1 with RPMI and incubated for 8 hours at 37 C with or without the presence of 10ng/ml of PMA and 1microg/ml of ionomycin (Sigma Chemical Co.)

[00106] ***Mitogen and antigen blastogenesis.*** Blood mononuclear cells are stimulated with mitogens or specific antigens to undergo cell division and proliferation. This process is monitored by measurement of thymidine incorporated into newly synthesized DNA within the cells. The mitogen which is used is Phytohaemagglutinin (PHA)(Difco, Detroit), in the working dilutions 1:25, 1:125, 1:625. Antigens will consist of Tetanus Toxoid (Connaught Laboratories Limited, Willowdale, Ontario), Candida (Miles Inc.), cytomegalovirus (CMV), herpes simplex virus (HSV), and varicella-zoster virus (VZV)(Myron J. Levin, M.D. UCHSC, Denver.CO). All reactions are run in triplicate with 10^5 cells plated per well. Incubation time for mitogen assays is 3 days and while that for antigen is 7 days, both at 37°C in 5% CO_2 . The cells are pulsed on the last day by adding 50ul of ^{3}H-Thymidine to each well for a final concentration of 1 uCi/well. The plates are harvested 6 to 18 hours after pulsing.

[00107] ***sPLA2:*** sPLA2 protein is measured using ELISA and sPLA2 activity using breakdown of thioester via methods previously described (61).

[00108] ***Serum levels of cytokines*** . We will use frozen serum samples to measure *TNF a, sIL-2R, IL-1, IL-2, IL-4, IL-6, IL-10, g-Interferon and CD40L* . A commercially available ELISA kit for cytokines is routinely used, according to the manufacturer' instructions (R&D Systems, Minneapolis and Immunotech, Westbrook, ME). ELISA kits for VCAM, ICAM and levels of sCD40L have recently become available from Chemicon, CA.

[00109] ***Genetic Markers.*** NO is synthesized in endothelial cells from L-arginine by the enzyme nitric oxide synthase (NOS) and there are known single nucleotide polymorphisms (SNPs) in the NOS3 gene. Since NO may play a key role in the regulation of bronchomotor

tone and inflammation of the airways (62), genetic studies evaluating the NOS gene in asthmatics may would be of interest. A method for rapidly genotyping multiple SNPs simultaneously has been developed at Roche Molecular Systems, Alameda, CA. An example of multiplex PCR products is shown in the agarose gel below. These 18 PCR products contain SNPs in genes thought to play a role in asthma: TNFα; CCqα; TNFR1: TNFβ; IL5Rα; TNFβ; IL9; CCR2; IL4Rα; CCR5: RMS1; β2AR; CC16; FcεRIβ; CTLA4; SCYA11; IL4Rα; IL4; and IL6.

EXAMPLE 1: ANALYSIS OF AMINO ACID LEVELS IN ASTHMATICS, SICKLE CELL DISEASE, AND PHT PATIENTS

[00110] Reductions were seen in plasma levels of many amino acids in asthmatic patient experiencing an acute exacerbation of respiratory symptoms (Table 1). Strikingly, the greatest decrease was in plasma levels of arginine, which were approximately half those of normal controls (45 ± 22 μM vs. 94 ± 29 μM; $p < 0.0001$).

Table 1. Plasma Amino Acids in Normal Controls vs. Asthma

Amino Acid	Concentration (μM)		% Control	p-value
	Controls (n = 15)	Asthma (n = 26)		
Arginine	94 ± 29	45 ± 22	48	< 0.0001
Ornithine	64 ± 21	49 ± 24	77	NS
Citrulline	30 ± 6	21 ± 10	70	0.002
Proline	195 ± 66	144 ± 73	74	0.03
Hydroxyproline	29 ± 14	19 ± 9	66	0.02
Lysine	162 ± 33	112 ± 57	69	0.004
Glutamic Acid	55 ± 29	40 ± 16	73	0.04
Glutamine	554 ± 86	466 ± 148	84	0.04
Glycine	251 ± 64	186 ± 103	74	0.03
Alanine	369 ± 104	292 ± 96	79	0.02
Valine	223 ± 52	161 ± 51	72	<0.001
Aspartic Acid	9 ± 6	7 ± 1	78	0.04
Threonine	136 ± 29	99 ± 58	73	0.02
Isoleucine	66± 20	48 ± 23	73	0.01
Leucine	126 ± 32	96 ± 45	76	0.03
Tyrosine	72 ± 15	52 ± 20	72	0.002
Histidine	75 ± 10	57 ± 20	79	0.003
Cysteine	22 ± 13	20 ± 16	90	NS
Asparagine	35 ± 15	41 ± 18 (n = 25)	118	NS
Serine	107 ± 32	89 ± 64	83	NS
Tryptophan	45 ± 10	37 ± 15	82	NS
Methionine	25 ± 6	20 ± 13	80	NS

Amino Acid	Concentration (μM)		% Control	p-value
	Controls (n = 15)	Asthma (n = 26)		
Phenylalanine	57 ± 13	56 ± 17	98	NS

Concentrations of amino acids are expressed as means ± SD. % Control values reflect percentages of controls for the asthma group.

[00111] As arginine, ornithine and lysine are taken up by cells via the same y+ transport system, the ratio arginine/(ornithine + lysine) provides an index of relative arginine availability at any given plasma arginine concentration. Relative arginine availability also was significantly lower in asthmatic patients as compared to normal controls (0.30 ± 0.13 vs. 0.42 ± 0.14, $p < 0.005$), further limiting arginine availability in the asthma group.

[00112] Plasma levels of ornithine (Table 1), a product of arginine catabolism, were generally lower in asthmatics relative to controls, and relative ornithine availability (ornithine/(arginine + lysine)) was somewhat higher in asthmatics than in controls (0.25 ± 0.07 for controls, 0.34 ± 0.17 for asthma), but neither of these trends reached statistical significance. On the other hand, citrulline, the precursor of endogenous arginine synthesis, was significantly reduced in asthmatics relative to normal controls (Table 1), possibly contributing to the decrease in plasma arginine levels in these patients.

[00113] Table 2 shows plasma amino acids in normal controls vs. patients with sickle cell disease (SCD). An abnormal amino acid profile is found in patients with sickle cell disease. The greatest deficiency is found in plasma arginine concentration.

Table 2: Plasma Amino Acids in Normal Controls vs. SCD

Amino Acid	Concentration (μM)		% Control	*p-value*
	Controls (n = 29)	*SCD (n = 163)*		
Nonessential:				
Arginine	**65 ± 16**	**40 ± 15**	**62**	**<0.0001**
***Ornithine**	61 ± 22	64 ± 23	--	NS
***Citrulline**	27 ± 11	25 ± 14	--	NS
***Proline**	**141 ± 49**	**205 ± 76**	**145**	**<0.0001**
***Glutamic acid**	**38 ± 15**	**47 ± 24**	**124**	**0.04**
Glutamine	**515 ± 129**	**607 ± 125**	**118**	**0.0004**
Glycine	**205 ± 48**	**278 ± 98**	**136**	**0.0001**
Tyrosine	**61 ± 13**	**53 ± 19**	**87**	**0.03**
Alanine	330 ± 69	321 ± 110	--	NS
***Cysteine**	40 ± 7	45 ± 15	--	NS
Serine	93 ± 15	94 ± 23	--	NS
Asparagine	44 ± 13	43 ± 14	--	NS
Essential:				
Lysine	**161 ± 30**	**143 ± 34**	**89**	**0.006**

Histidine	73 ± 15	56 ± 16	77	<0.0001
Phenylalanine	61 ± 13	53 ± 19	87	0.03
*Leucine	114 ± 25	89 ± 28	78	<0.0001
*Valine	207 ± 41	162 ± 45	78	<0.0001
Isoleucine	58 ± 13	49 ± 16	84	0.008
Methionine	25 ± 5	26 ± 7	--	NS
Threonine	137 ± 31	126 ± 45	--	NS

Concentrations of amino acids are expressed as means ± SD.

% Control: Values are shown only when significantly different from controls.

*Amino acids that are altered in SCD patients with PHT vs. SCD patients without PHT

[00114] Table 3 illustrates plasma amino acid levels that differ in sickle cell disease patients with pulmonary hypertension compared to those without pulmonary hypertension. Elevated downstream by-products of arginase activity occur in SCD patients who have developed pulmonary hypertension.

Table 3: Plasma Amino Acids in SCD with PHT vs. SCD with PHT

Amino Acid	Concentration (μM)			*p-value*
	Controls (n=29)	*TR jet < 2.5 (n=86)*	*TR jet ≥ 2.5 (n=41)*	*(PHT vs non PHT)*
Nonessential:				
Ornithine	61 ± 22	59 ± 20	69 ± 23	0.02 (↑)
Citrulline	27 ± 11	*22 ± 10	29 ± 20	0.008 (↑)
Proline	141 ± 49	*192 ± 74	*236 ± 87	0.003 (↑)
Glutamic acid	38 ± 15	*45 ± 16	*60 ± 37	0.003 (↑)
Cysteine	40 ± 7	43 ± 14	*48 ± 16	0.04 (↑)
Essential:				
Valine	207 ± 41	*165 ± 41	*145 ± 48	0.01 (↓)
Leucine	114 ± 25	*92 ± 25	*78 ± 30	0.006 (↓)

Concentrations of amino acids are expressed as means ± SD.

*Amino acids that differ significantly ($p<0.05$) from controls

EXAMPLE 2: ARGININE AND ARGINASE LEVELS IN ASTHMATIC PATIENTS AND SICKLE CELL DISEASE (SCD) PATIENTS WITH PULMONARY HYPERTENSION

[00115] SCD and asthmatic patients exhibited a significant arginine deficiency during acute exacerbations. Serum arginine levels are summarized in the table below, and presented in Figure 1 (Panel A).

	Normal	SCD with PHT	Asthma
Serum arginine (μM)	109.0±33.1	55.4±16.0	38.9±20

PHT = pulmonary hypertension; $p<0.0001$ for comparison of SCD with PHT vs. normal, and for asthma vs. normal.

[00116] Arginase activity was elevated in SCD patients with PHT relative to normal controls, and was even greater in asthmatic patients. Serum arginase activity levels are summarized in the table below, and the data presented in Figure 1 (Panel B).

	Normal	SCD with PHT	Asthma
Serum arginase activity (μmol/ml/hr)	0.427±0.2	0.95±0.7	1.6±0.9

$p=0.001$ for comparison of SCD with PHT vs. normal, and for asthma vs. normal.

[00117] Figure 1 (Panel B) is a graph showing arginase activity in normal non-asthmatic controls (Normal, n=10) vs. patients with sickle cell disease and pulmonary hypertension (SCD, n=17) vs. patients with asthma (Asthma, n=20). Arginase activity was significantly increased in patients with asthma compared to normal controls ($p<0.001$). Arginase activity is even higher in asthmatics compared than sickle cell patients with pulmonary hypertension. Two patients with SCD having the highest levels of arginase activity died within 1 year of obtained values. Elevated arginase activity may be a reflection of increased disease severity in sickle cell disease, and is likely an inflammatory marker in asthma that potentially plays a role in disease pathogenesis.

[00118] As illustrated in Figure 2, arginine levels rose significantly by discharge in asthmatics admitted to the hospital ((54.7±29 vs. 93.1±37 μM, $p<0.05$, n = 4). Serial arginase activity levels were available on two patients and dropped substantially by discharge in each case (1.85 decreased to 1.12 μmol/ml/hr and 3.86 decreased to 0.50 μmol/ml/hr). It is likely that high arginase activity in asthmatic patients contributes to low circulating arginine levels, thereby limiting arginine bioavailability and creating a nitric oxide deficiency that induces hyperreactive airways.

[00119] Figure 3 represents changes in plasma arginine and ornithine concentration, arginase activity and nitric oxide metabolites during hospitalization in a representation four-year old boy with status asthmaticus. Sequential plasma arginine (*filled circles*) and ornithine levels (*unfilled circles*) are followed over a three-day hospitalization. Day "*1*" is the day of admission, obtained in the emergency department, and day "*3*" is the day of discharge. As shown in Panel A of Figure 3, low arginine levels increase significantly during the course of hospitalization, as does the arginine-to-ornithine ratio (0.65, day 1 vs. 1.6, day 2 vs. 1.9, day 3).

[00120] As shown in Panel B of Figure 3, serum nitric oxide metabolites (*unfilled circles*) and arginase activity (*filled circles*) are also followed over the three-day hospitalization. Arginase activity dropped dramatically as the patient clinically improved, and reached a normal level by discharge, corresponding to an increase in serum nitric oxide metabolite production. An improvement in arginine and nitric oxide bioavailability occurred as the asthma exacerbation resolves.

[00121] In addition, the inflammatory state of the patient's condition can also play a role, as arginase gene expression is upregulated by many cytokines involved in the inflammatory process, particularly the Th2 cytokines. Elevated sPLA2 levels were observed in asthmatic patients vs. normal controls (4.2±2 vs. 25.9±30, $p<0.05$, normal control vs. asthma) in serum. Since phospholipase A2 is a precursor to leukotrienes, elevated sPLA2 may identify patients who would respond to leukotriene inhibitors. Combination therapy of one or more agents described herein with leukotriene inhibitors or sPLA2 inhibitors/antibodies is beneficial for patients with asthma and other inflammatory conditions involving elevated cytokines.

EXAMPLE 3: ARGINASE LEVELS OF SCD PATIENTS WITH PHT AFTER TREATMENT WITH ARGININE

[00122] Patients with sickle cell disease and documented pulmonary hypertension by echocardiography were treated with oral L-arginine-HCl, at a dose of 0.1g/kg TID for five days. Echocardiograms were performed before and after L-arginine administration, on Day 0 and Day 6, and at ≥ 1one month follow-up after completion of arginine therapy. Blood samples for determination of amino acid levels were drawn in the morning of Day 0 (pre-treatment), Day three3, and Day six of the study. Arginase activity levels were determined on Day 0. No patients were being concurrently treated with vasodilators or anticoagulant agents, and no patients received a red blood cell transfusion during the five-day study period. Cardiologists involved in the interpretation of echocardiograms were unaware of the therapy given.

[00123] *Echocardiography.* Oral L-arginine supplementation significantly reduced pulmonary artery systolic pressure by a mean of 15.2% (63.9±13 to 54.2±12 mmHg, $p=0.002$) after five days of therapy. One patient was determined to be non-compliant based on plasma L-arginine concentration at the end of the study (61.5 μM/L at Day 0 vs. 44.9μM/L Day 6). He was the only patient found to not show an improvement in pulmonary hypertension by echocardiogram.

[00124] The tricuspid regurgitant jet velocity from echocardiograms obtained > two months prior to study enrollment demonstrated stable estimated pulmonary artery systolic pressures in five5 patients, and worsening pulmonary hypertension in two patients. Results were unavailable from outside hospitals in three patients. Follow-up echocardiography was obtained at ≥ one month after arginine therapy in the nine compliant patients, with mixed results. The non-compliant patient was lost to follow-up. Four patients reverted to their previous baseline pulmonary artery systolic pressures, four patients exhibited persistent improvement, and one patient demonstrated a worsening of pulmonary hypertension (echocardiography done while admitted for acute chest syndrome). Two of the patients demonstrating persistent improvement had also been started on transfusion therapy due to the severity of their disease, and one of these two patients had continued arginine therapy (at a dose of 0.1 gm/kg BID).

[00125] *Amino Acid levels.* Plasma L-arginine levels were low in patients with pulmonary hypertension compared to normal controls (50.8±19 vs. 114±27μM, $p<0.0001$), but similar to levels found in sickle cell patients at steady-state who did not have pulmonary hypertension. However the arginine-to-ornithine ratio was significantly lower in patients with pulmonary hypertension compared to normal controls (0.95±0.3 vs 2.0±0.6, $p<0.0001$), suggesting increased arginase activity and decreased arginine bioavailability. Both L-arginine and ornithine concentrations increased significantly after five days of oral L-arginine supplementation ($n=10$, $p<0.05$).

[00126] *Arginase activity.* Arginase converts L-arginine to ornithine and urea. Arginase activity in serum was higher in sickle cell patients with pulmonary hypertension compared to normal controls (0.82±0.6 vs. 0.43±0.2μmol/ml/h). Of interest, the patients with the two highest levels of arginase activity (1.22 and 2.46μmol/ml/h) have died within one year of enrollment. Elevated arginase activity may be a marker for disease severity.

EXAMPLE 4: L-ARG AND NOHA COMBINATION THERAPY

[00127] The effect of L-Arg and the arginase inhibitor NOHA, alone and in combination in the treatment of SCD is examined. The effect agents are examined on cell sickling, red cell indices, on functional properties of hemoglobin and on the existence of adverse effects such as hemoglobin oxidation and red cell hemolysis. The effect of the agents on interactions between sickle cells and endothelial cells, membrane transport properties and cell volume control are also examined. *In vivo* studies are performed using various lines of transgenic mice which

produce different levels of Hemoglobin S, including those which produce human Hb S/Hb F exclusively.

EXAMPLE 5: ARGININE MONOTHERAPY AND COMBINATION THERAPY OF ARGININE AND MAGNESIUM

[00128] A randomized, double-blinded placebo-control trial of intravenous arginine or arginine and magnesium for the treatment of status asthmaticus is conducted as follows. Patients with respiratory distress and asthma are recruited from the emergency department or clinics at Children's Hospital Oakland. Study drug is administered as a one-time dose in the emergency department. Arginine or placebo is continued every 8 hours for patients admitted to the hospital. Primary outcome measures are admission vs. discharge patient parameters, and length of hospital stay, improvement in clinical asthma scores and oxygen saturations/need for supplemental oxygen use. Plasma amino acids, arginine-to-ornithine ratio, arginase activity, nitric oxide metabolites (in serum, exhaled breath and urine), PLA2, cytokines, inflammatory biomarkers, genetic modifiers and peak flows are followed.

EXAMPLE 6: ARGININE MONOTHERAPY

[00129] Although Chambers et al. "Effect of nebulised L- and D-arginine on exhaled nitric oxide in steroid naive asthma." Thorax. 2001 Aug;56(8):602-6. reported that administration of inhaled L-Arg to asthma patients induced bronchoconstriction, with exhaled NO decreasing with acute bronchoconstriction, and returning to baseline with the resolution of bronchoconstriction, similar bronchoconstriction occurred with their control test using an alternate amino acid. It is likely that the acute bronchoreaction was due to irritation of the inhalant itself, rather than arginine. Irritation can be avoided by careful selection of a non-irritating inhalant and/or selection of formulation components that do not cause significant irritation upon inhalation (i.e., a low irritant or non-irritating formulation). Such issues can also be avoided by administration of arginine by a route other than inhalation, e.g., by oral or intravenous administration.

[00130] The effects of arginine supplementation on pulmonary function tests is evaluated by administering supplemental arginine (oral or intravenous) alone or in conjunction with magnesium and/or an arginase inhibitor to patients with a known diagnosis of asthma, defined as ≥ 3 wheezing episodes and a past history of asthma medication usage (e.g., bronchodilators, steroids, inhaled steroids, or leukotriene inhibitors etc). Pulmonary function tests are performed before and after a single dose of arginine (0.1 gram/kg to a max of 10 grams).

[00131] One patient has already been enrolled in this study. A single dose of oral arginine (0.1gm/kg) was administered. Pulmonary function tests were determined prior to treatment and 2 hours after arginine supplementation. Although supplemental arginine did not significantly effect spirometry (except FIF 50% - inducing a 23% improvement), and had minimal effect on lung volumes, treatment had an impressive impact on airway resistance within 2 hours (*Raw* decreased by 22% and *Gaw* increased by 28%). Since increased airway resistance is a significant problem during an acute exacerbation of asthma, a benign therapy that decreases airway resistance, likely through smooth muscle relaxation, benefits patients with asthma. Also of interest, the patient's oxygen saturation by venous blood gas increase from 85 to 92%.

[00132] Even greater benefits can appreciated after more than 2 hours post treatment or when used in combination with standard of care asthma therapy such as bronchodilators and steroids.

REFERENCES CITED

1. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nat Med 1987; 327:524-526.

2. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993; 329:2002-2012.

3. Kam PC, Govender G. Nitric oxide: Basic science and clinincal applications. Anaesthesia 1994; 49:515-521.

4. Zoritch B. Nitric oxide and asthma. Arch Dis Child 1995; 72:259-262.

5. Gaston B, Drazen JM, Loscalzo J, Stamler J. The biology of nitrogen oxides in the airways. Am J Respir Crit Care Med 1994; 149:538-551.

6. Nathan C, Shiloh M. Reactive oxygen and nitrogen intermediates in the relationship between
mammalian hosts and microbial pathogens. Proc Natl Acad Sci 2000; 97:8841-8848.

7. Xia Y, Dawson V, Dawson T, Snyder S, Zweier J. Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. Proc Natl Acad Sci 1996; 93:6770-6774.

8. Dias-Da-Motta P, V. A, Muscara M, Saad S. The release of nitric oxide and superoxide anion by neutrophils and mononuclear cells from patients with sickle cell anaemia. Brit J Haematol 1996; 93:333-340.

9. Demiryurek A, Dakici I, Danzik I. Peroxynitrite: A putative cytotoxin. Pharm Toxicology 1998; 82:113-117.

10. Bowton D, Seeds M, Fasano M, Goldsmith B, Bass D. Phospholipase A2 and arachidonate increase in bronchoalveolar lavage fluid after inhaled antigen challenge in asthmatics. Am J Respir Crit Care Med 1997; 155:421-425.

11. Holgate S. Asthma genetics: waiting to exhale. Nat Genet 1997; 15:227-229.

12. Hamid Q, Springall DR, Riveros-Morena V, Chanez P, Howarth P, Redington A, Bousquet J, Godard P, Holgate S, Polak JM. Induction of nitric oxide synthase in asthma. Lancet 1993; 342:1510-1513.

13. Nijkamp FP, Folkerts G. Nitric oxide and bronchial hyperresponsiveness. Arch Int Pharmoocodyn 1995; 329:81-96.

14. Kharitonov SA, Yates D, Barnes PJ. Increased nitric oxide in exhaled air of normal human subjects with upper respiratory tract infections. Eur Respir J 1995; 8:295-7.

15. Ricciardolo F, Geppetti P, Mistretta A, Nadel J, Sapienza M, Bellofiore S, Di Maria G. Randomized double-blind placebo-controlled study of the effect of inhibition of nitric oxide synthesis in bradykinin-induced asthma. Lancet 1996; 348:374-377.

16. Meurs H, Schuurman F, Duyvendak M, Zaagsma J. Deficiency of nitric oxide in polycation-induced airway hyperreactivity. Br J Pharmacol 1999; 126:559-562.

17. Sanders S. Nitric oxide in asthma. Am J Respir Cell Mol Biol 1999; 21:147-149.

18. Pieper GM. Review of Alterations in Endothelial Nitric Oxide Production in Diabetes. Protective Role of Arginine on Endothelial Dysfunction. Hypertension 1998; 31:1047-1060.

19. Lerman A, Burnett JC, Higano ST, McKinley LJ, Holmes DR. Long-term L-arginine supplementation improves small-vessel coronary endothelial function in human. Circulation 1998; 97:2123-2128.

20. Perrine SP, Ginder GD, Faller DV, Dover GH, Ikuta T, Witkowska HE, Cai SP, Vichinsky EP, Olivieri NF. A short-term trial of butyrate to stimulate fetal-globin-gene expression in the beta-globin disorders. N Engl J Med 1993; 328:81-6.

21. Maxwell AJ, Cooke JP. 1998. Cardiovascular effects of L-arginine. *In* P. Vallance and C. Baylis, editors. Current Opinion in Nephrology and Hypertension. 133.

22. Creager MA, Gallagher SJ, Girerd XJ, Coleman SM, Dzau VJ, Cooke JP. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. J Clin Invest 1992; 90:1248-53.

23. Drexler H, Zeiher AM, Meinzer K, Just H. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. Lancet 1991; 338:1546-50.

24. Folkerts G, Van der Linde HJ, Nijkamp FP. Virus-induced airway hyperresponsiveness in guinea pigs is related to a deficiency in nitric oxide. J Clin Invest 1995; 94:26-30.

25. Solomons C, Cotton CK, Dubois R. The use of buffered L-arginine in the treatment of cystic fibrosis. Pediatr 1971; 47:384-390.

26. Solomons C, Hathaway W, Cotton E. L-arginine, the sickling phenomenon, and cystic fibrosis. Pediatr 1972; 49:933.

27. Knight J, Murphy TM, Browning I. The lung in sickle cell disease. Pediatr Pulmonol 1999; 28:205-216.

28. Gladwin M, Schechter A. Nitric oxide therapy in sickle cell disease. Semin Hematol 2001; 38:333-342.

29. Lopez da Mata P, Neuparth N, Carmo M, Caires I, Macedo P, Rendas A. 1998. How does nitrates in blood correlated to exhaled levels in asthma? . European Respiratory Conference, Geneva, Switzerland.

30. Rees DC, Cervi P, Grimwade D, O'Driscoll A, Hamilton M, Parker NE, Porter JB. The metabolites of nitric oxide in sickle-cell disease. Br J Haematol 1995; 91:834-7.

31. Morris CR, Kuypers FA, Larkin S, Vichinsky E, Styles L. Patterns of arginine and nitric oxide in sickle cell disease patients with vaso-occlusive crisis and acute chest syndrome. J Pediatr Hematol Oncol 2000; 22:515-520.

32. Minter K, Gladwin M. Pulmonary complications of sickle cell amemia. A need for increased recognition, treatment, and research. Am J Respir Crit Care Med 2001; 164:2016-2019.

33. Morris CR, Kuypers FA, Larkin S, Sweeter N, Simon J, Vichinsky EP, Styles L. Arginine therapy: A novel strategy to increase nitric oxide production in sickle cell disease. Brit J Haematol 2000; 111:498-500.

34. Morris C, Morris S, Jr., Hagar W, van Warmerdam J, Claster S, Kepka-Lenhart K, Machado L, Kuypers F, Vichinsky E. Arginine Therapy: A new treatment for pulmonary hypertension in sickle cell disease? Am J Respir Crit Care Med 2003; 168:63-69.

35. Boucher JL, Moali C, Tenu JP. Nitric oxide biosynthesis, nitric oxide synthease inhibitors, and arginase competition for L-arginine utilization. Cell Mol Life Sci 1999; 55:1015-1028.

36. Morris SM, Jr. , Kepka-Lenhart D, Chen L. Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. Am J Physiol Endocrinol Metab 1998; 275:740-747.

37. Mori M, Gotoh T. 2000. Relationship between arginase activity and nitric oxide production. *In* L. Ignarro, editor. *Nitric Oxide. Biology and Pathology.* Academic Press, San Diego. 199-208.

38. Waugh W, Daeschner C, Files B, Gordon D. Evidence that L-arginine is a key amino acid in sickle cell anemia - a preliminary report. Nutritional Research 1999; 19:501-518.

39. Meurs J, McKay S, Maarsingh H, Hamer M, Macic L, Molendijk N, Zaagsma J. Increased arginase activity underlies allergen-induced deficiency of cNOS-dervied nitric oxide and airway hyperresponsiveness. Br J Pharmacol 2002; 136:391-398.

40. Morris CR, Kuypers A, Vichinsky E, Kepka-Lenhart D, Morris SM, Jr. 2002. Elevated serum arginase activity in patients with sickle cell disease and pulmonary hypertension. . The 30th Anniversary of the National Sickle Cell Program, Washington, DC.

41. Morris SM, Jr. Regulation of enzymes of the urea cycle and arginine metabolism. Annu Rev Nutr 2002; 22:87-105.

42. Morris SM, Jr. 2000. Regulation of arginine availability and its impact on NO synthesis. . Nitric Oxide. Biology and Pathobiology. Academic Press, San Diego. 187-197.

43. Mori M, Gotoh T. Regulation of nitric oxide production by arginine metabolic enzymes. Biochem Biophys Res Commun 2000; 275:715-719.

44. Kershenobich D, Fierro F, Rojkind M. The relationship between the free pool of proline and collagen content in human liver cirrhosis. J Clin Invest 1970; 49:2246-2249.

45. Albina J, Abate J, Mastrofrancesco B. Role of ornithine as a proline precursor in healing wounds. J Surg Res 1993; 55:97-102.

46. Endo M, Oyadomari S, Terasaki Y, Takeya M, Suga M, Mori M, Gotoh T. Induction of arginase I and II in bleomycin-induced fibrosis of mouse lung. Am J Physiol Lung Cell Mol Physiol 2003; 285:L313-L321.

47. Tanaka H, Masuda T, Tokuoka S, Komai M, Nagao K, Takahashi Y, Nagai H. The effect of allergen-induced airway inflammation on airway remodeling in a murine model of allergic asthma. Inflamm Res 2001; 50:616-624.

48. Elias J, Zhu Z, Chupp G, Homer R. Airway remodeling in asthma. J Clin Invest 1999; 104:1001-1006.

49. Elias J, Lee C, Zheng T, Ma B, Homer R, Zhu Z. New insights into the pathogenesis of asthma. J Clin Invest 2003; 111:291-297.

50. Busse W, Lemanske R. Asthma. N Engl J Med 2001; 344:350-362.

51. Kurosawa M, Shimizu Y, Tsukagoshi H, Ueki M. Elevated levels of peripheral-blood, naturally occurring aliphatic polyamines in bronchial asthmatic patients with active symptoms. Allergy 1992; 47:638-643.

52. Sward K, Pato M, Nilsson B, Nordstrom I, Hellstrand P. Polyaminines inhibit myosin phosphatase and increase LC20 phosphorylation and force in smooth muscle. Am J Physiol 1995; 269:C563-C571.

53. Nilsson B, Hellstrand P. Effects of polyamines on intracellular calcium and mechanical activity in smooth muscle of guinea-pig taenia coli. Acta Physiol Scand 1993; 148:37-43.

54. Hoet P, Nemery B. Polyamines in the lung: polyamine uptake and polyamine-linked pathological or toxicological conditions. Am J Physiol Lung Cell Mol Physiol 2000; 278:L417-L433.

55. Stuehr DJ, Kwon N, Nathan CF, Griffith OW, Felman PL, Wiseman J. N-Hydroxyl-L-arginine is an intermediate in the biosynthesis of nitric oxide for L-arginine. J Biol Chem 1991; 266:6259-6263.

56. Kumar A, Brar R, Wang P, Dee L, Skorupo G, Khadour F, Schulz R, Parrillo J. Role of nitric oxide and cGMP in human septic serum-induced depression of cardiac myocyte contractility. Am J Physiol 1999; 276:265.

57. Zeballos A, Bernstein R, Thompson C, Forfia P, Seyedi N, Kaminiski R, Wolin M, Hintze T. Pharmocodynamics of plasma nitrate/nitrite as an indication of nitric oxide formation in conscious dogs. Circulation 1995; 91:2982.

58. Miller V, Lewis D, Rud K, Offord K, Croghan I, Hurt R. Plasma nitric oxide before and after smoking cessation with nicotine nasal spray. J Clin Pharmacol 1998; 38:22.

59. Nelson BV, Sears S, Woods J. Expired nitric oxide as a marker for childhood asthma. J Pediatr 1997; 130:423-427.

60. Baraldi E, Azzolin NM, Zanconato S, Dario C, Zacchello F. Corticosteriods decrease exhaled nitric oxide in children with acute asthma. J Pediatr 1997; 131:381-385.

61. Styles LA, Schalkwijk CG, Aarsman AJ, Vichinsky EP, Lubin BH, Kuypers FA. Phospholipase A2 levels in acute chest syndrome of sickle cell disease. Blood 1996; 87:2573-8.

62. Li J. Mechanisms of asthma. Current Opinions in Pulmon Med 1997; 3:10-16.

[00133] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and

scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

What is claimed is:

1. A method of treating a subject having elevated arginase as a symptom or cause of a disorder, the method comprising:

administering to a subject in need of therapy an amount of L-arginine and an amount of an arginase inhibitor, said administering being effective to enhance arginine bioavailability in the subject thereby treating the subject.

2. The method of claim 1, wherein the subject is an asthmatic.

3. The method of claim 1, wherein the subject has sickle cell disease.

4. The method of claim 1, wherein the subject has pulmonary hypertension.

5. The method of claim 1, wherein the arginase inhibitor is N(omega)-hydroxy-nor-L-arginine (NOHA), N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) , 2(S)-amino-6-boronohexanoic acid (ABH), S-(+)-Amino-6-iodoacetamidohexanoic acid, S-(+)-Amino-5-iodoacetamidopentanoic acid, L-norvaline, or L-HOArg.

6. The method of claim 1, wherein the arginase inhibitor is NOHA.

7. The method of claim 1, wherein said administering further comprises administering an amount of nitric oxide (NO).

8. A method of treating asthma symptoms in a subject, the method comprising:

administering L-arginine to a subject having or at risk of asthma, said administering being effective to enhance arginine bioavailability in the subject thereby treating asthma symptoms in the subject.

9. The method of claim 8, wherein the method further comprises administration of magnesium.

10. The method of claim 8, wherein the method further comprises administration of an arginase inhibitor.

11. A method of treating pulmonary hypertension in a subject, the method comprising:

administering L-arginine and an arginase inhibitor to a subject having or at risk of pulmonary hypertension, said administering being effective to enhance arginine bioavailability in the subject thereby treating asthma symptoms in the subject.

12. The method of claim 11, wherein the subject has sickle cell disease.

13. The method of claims 11 or 12, wherein the arginase inhibitor is N(omega)-hydroxy-nor-L-arginine (NOHA), N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) , 2(S)-amino-6-boronohexanoic acid (ABH), S-(+)-Amino-6-iodoacetamidohexanoic acid, S-(+)-Amino-5-iodoacetamidopentanoic acid, L-norvaline, or L-HOArg.

14. The method of claims 11 or 12, wherein the arginase inhibitor is NOHA.

15. The method of claims 11 or 12, wherein said administering further comprises administering an amount of nitric oxide (NO).

16. A method of treating a subject having decreased nitric oxide bioavailability as a symptom or cause of a disorder, the method comprising:

administering to a subject in need of therapy an amount of L-arginine and an amount of magnesium, said administering being effective to enhance arginine bioavailability in the subject thereby treating the subject.

17. The method of claim 16, wherein the subject is an asthmatic.

18. The method of claim 16, wherein the subject has sickle cell disease.

19. The method of claim 16, wherein the subject has pulmonary hypertension.

20. The method of claim 16, wherein the decreased nitric oxide bioavailability is a result of decreased arginine in the subject.

21. A composition comprising L-arginine, an arginase inhibitor, and a pharmaceutically acceptable excipient.

22. A composition comprising L-arginine and a pharmaceutically acceptable magnesium salt, and a pharmaceutically acceptable excipient.

FIG. 1

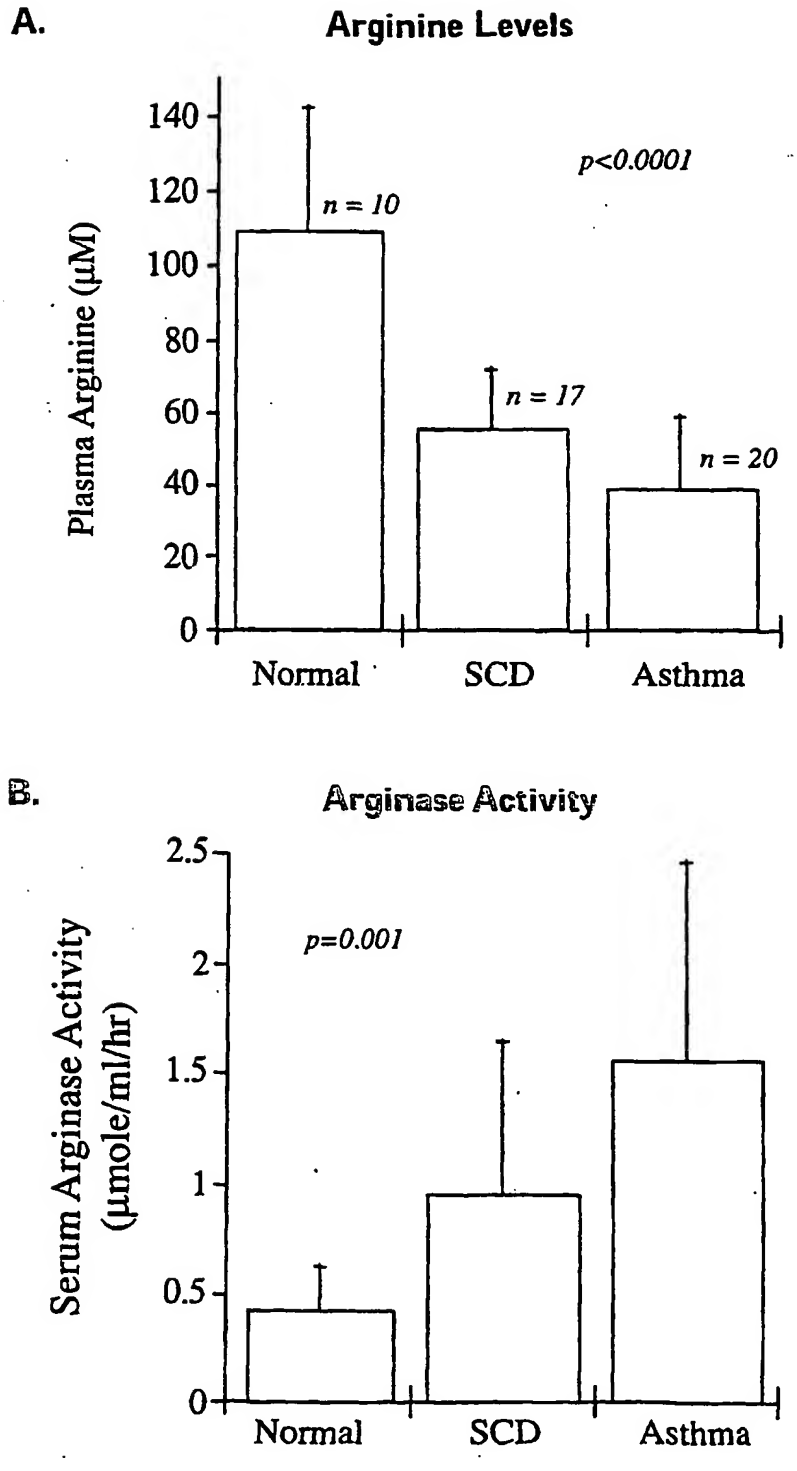

FIG. 2

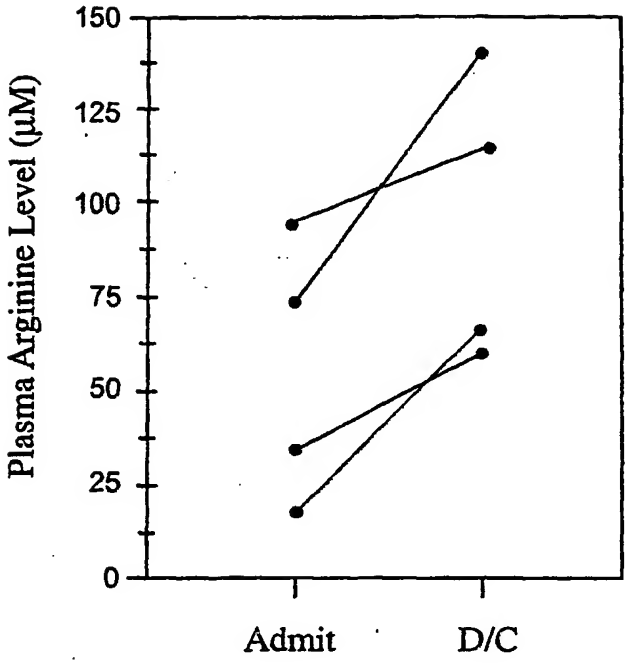

FIG. 3

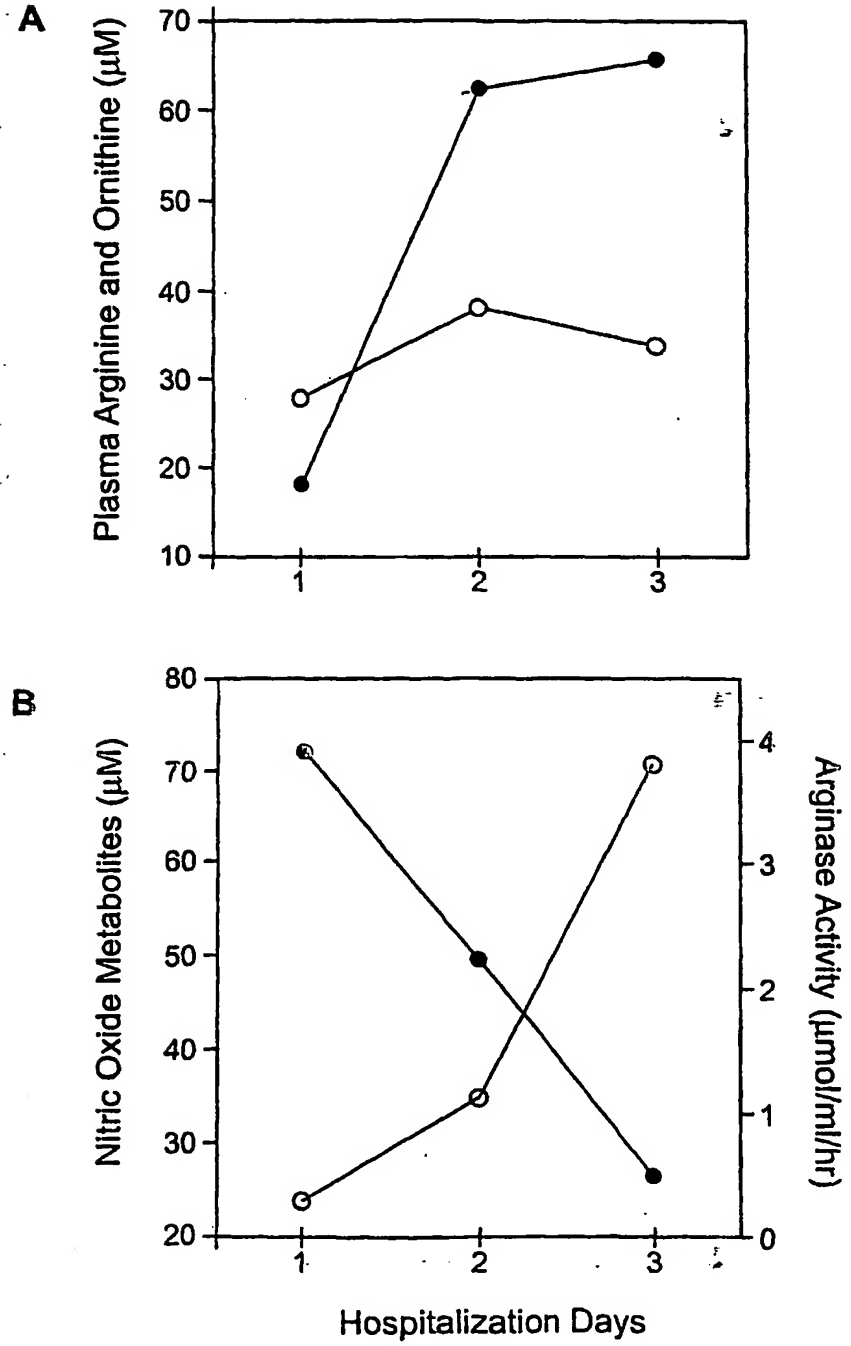

FIG. 4

Arginase competes with NOS for L-Arg